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HMGI(Y) and Sp1 in addition to NF-κB regulate transcription of the MGSA/GROα gene

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ABSTRACT

Expression of the chemokine MGSA/GRO is upregulated as melanocytes progress to melanoma cells. We demonstrate that constitutive and cytokine induced MGSA/GROα expression requires multiple DNA regulatory regions between positions –143 to –62. We have previously shown that the NF-κB element at –83 to –65 is essential for basal and cytokine induced MGSA/GROα promoter activity in the Hs294T melanoma and normal retinal pigment epithelial (RPE) cells, respectively. Here, we have determined that the Sp1 binding element located ~42 base pairs upstream from the NF-κB element binds Sp1 and Sp3 constitutively and this element is necessary for basal MGSA/GROα promoter activity. We demonstrate that the high mobility group proteins HMGI(Y) recognize the AT-rich motif nested within the NF-κB element in the MGSA/GROα promoter. Loss of either NF-κB or HMGI(Y) complex binding by selected point mutations in the NF-κB element results in decreased basal and cytokine induced MGSA/GROα promoter activity. Thus, these results indicate that transcriptional regulation of the chemokine MGSA/GROα requires at least three transcription factors: Sp1, NF-κB and HMGI(Y).

INTRODUCTION

The initiation of gene transcription is accomplished through a cascade of events involving sequence-specific DNA-binding proteins. Many of these *trans*-activating proteins are expressed in a cell type specific manner, though ubiquitously expressed *trans*-activators can contribute to selective cell specific expression. An additional level of transcriptional regulation has recently been revealed whereby several *trans*-activators from structurally distinct families interact with each other. These interactions bestow a higher level of specificity to the expression of genes containing these multiple regulatory elements.

The ubiquitous eukaryotic Sp1 complex was initially identified as a transcription factor that bound to GC-rich promoter elements found in numerous cellular and viral promoters (1,2). There have been several Sp-related factors identified including Sp1, Sp3 and Sp4, all of which contain glutamine-rich and serine/threonine-rich domains (3,4). Sp3 is similar to Sp1 in that it recognizes Sp1 consensus elements in several promoters, including c-myc, uteroglobin, and the HIV-1 and SV40 enhancers (5–8). Since Sp3 lacks an activation domain, it serves as a repressor of Sp1 activity by competitively binding to Sp1 consensus elements (5,7,8). In contrast, other groups have demonstrated that Sp3 is a positive transcriptional regulator, though less potent than Sp1 (6).

Sp1 has been demonstrated to interact with the N-terminal portion of several NF-κB subunits (9,10). Furthermore, Sp1 interacts through its glutamine-rich activating domains with components of the general transcriptional machinery, including the TATA box-binding protein (TBP) and the co-activator TAF110 (11–13). The retinoblastoma (Rb) protein has been demonstrated to interact with both Sp1 and Sp3 to 'superactivate' promoters containing retinoblastoma control elements (RCE) (6,14,15). The Sp1 heterotypic interactions with such binding proteins, as well as homotypic interactions, have been postulated to occur either directly or through the Sp1 complex bending and looping out intervening DNA (9,16–18).

Several transcription accessory factors in addition to Sp1 have been identified that bend DNA to facilitate the formation of these higher order nucleoprotein complexes (19,20). Such accessory factors include the high mobility group proteins HMGI(Y) which are non-histone chromosomal proteins that specifically recognize the minor groove or AT-rich portions of DNA (21–25). HMGI(Y) levels are elevated in rapidly growing, undifferentiated and transformed cells (26–28). HMGI(Y) has been postulated to interact with DNA major groove binding *trans*-activators through its acidic C-terminal domain (25,29). Such interactions contribute to the stabilization of protein–protein complexes bound to their consensus elements. This has been demonstrated for the IFN-β and E-selectin promoters, where induction requires cooperativity between the HMGI(Y), NF-κB and ATF-2 transcriptional complexes. HMGI(Y) increases the affinity of NF-κB or ATF-2 to the IFN-β PRDII and PRDIV elements (30,31) and to several NF-κB and adjacent elements in the E-selectin gene (32,33). Interestingly, the MGSA/GRO genes have several DNA regulatory elements whose binding factors have been implicated in DNA bending and constitutive transactivation including Sp1, C/EBP-like, NF-κB and putative HMGI(Y) binding elements. The closely related IL-8 gene is similar with the exception that an AP-1 consensus element is located where the Sp1 element is found within MGSA/GROα (34,35).

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The chemokines MGSA/GRO and IL-8 play a major role in mediation of inflammation and wound healing as potent chemoattractants for neutrophils (36–40,41 for review). Altered expression of these chemokines appears to be enhanced in several inflammatory and tumorigenic processes (41–43 for review). MGSA/GRO expression is upregulated as melanocytes progress to malignant melanoma (44–46). Furthermore, both MGSA/GRO and IL-8 serve as autocrine growth factors for several melanoma cell lines (37,44,47–49). The murine MGSA/GRO homolog MIP-2 exhibits constitutive transcription in B16 melanoma cells (50). The NF-κB element located ~40 base pairs (bp) from the TATA box region is a major contributor to the basal and cytokine induced MGSA/GROα and MGSA/GROβ promoter activity (51–53).

The work described here characterizes the role of the Sp1 and HMGI(Y) binding elements in addition to the NF-κB element in MGSA/GROα transcriptional regulation in both normal retinal pigment epithelial (RPE) cells and the Hs294T melanoma cell line. We show that both Sp1 and Sp3 constitutively recognize the Sp1 consensus element in the MGSA/GROα immediate promoter and contribute to the basal MGSA/GROα expression. HMGI(Y) also binds constitutively to the five nucleotide AT-rich region nested within the NF-κB element in addition to the NF-κB p50 and NF-κB p65 heterodimer complex. Loss of either HMGI(Y), NF-κB or Sp1 results in decreased MGSA/GROα promoter activity, suggesting that these complexes collectively contribute to the transcriptional regulation of the chemokine MGSA/GROα.

MATERIALS AND METHODS

CAT reporter gene and expression vector constructs

MGSA/GROα deletion promoter and mutant NF-κB MGSAα350/CAT constructs were described earlier (51). Mutation of the NF-κB element in the AT-rich region in MGSAα350/CAT was achieved by recombinant PCR using the primer mAT-2 5′-CTGGAACTCCGGGATTCCCCTGGCC-3′ and its complement. Further deletion promoter constructs of the MGSA/GROα promoter were made by subjecting MGSAα350/CAT and mutant NF-κB MGSAα350/CAT to Exonuclease III digestion to generate a series of 5′ deletions of MGSA/GROα promoter from the *Hin*dIII site in MGSAα350/CAT (Erasabase, Promega).

Site directed mutagenesis was utilized to generate mutations within the Sp1 and NF-κB elements using the Altered Sites *in vitro* Mutagenesis System (Promega). Briefly, the MGSA/GROα 350 bp promoter fragment was subcloned into pALTER-1. The resulting plasmid was subjected to mutagenesis using the oligonucleotides mGC-4B, mAT-B and MGSAαmSp1 described below. The expected point mutations were confirmed by restriction enzyme digestion and sequencing. The MGSA/GROα 350 bp fragment containing the point mutations was then subcloned into the *Hin*dIII site of pPLFCAT (54,55). The NF-κB p65 expression vector was as previously described (56).

Transfection and CAT reporter assay

Hs294T cells were obtained from American Type Culture Collection (ATCC) and cultured as described (51). Retinal pigment epithelial (RPE) cells were cultured as described (51,57). Both cell types were co-transfected with 10 μg of the indicated MGSA/CAT fusion genes and 2 μg of pCMVhGH (obtained from Dr Lynn Matrisian, Vanderbilt University) which allows for normalization of transfection efficiency by measuring growth hormone (GH) secretion by immunoassay (Nichols Institute). Transfections were performed by the calcium phosphate co-precipitation method (58). CAT enzymatic activity was assayed as previously described (54). The percent [^{14}C]-chloramphenicol converted to acetylated forms was determined by phosphoimage analysis (Molecular Dynamics).

Table 1. Sequences of oligonucleotides used in gel mobility shift analyses and site-directed mutagenesis

IgG κB	5′-	TCG ACA GAG GGG ACT TTC CGC AGA GGC -3′
IL-8 -101/-63	5′-	G GGC CAT CAG TTG CAA ATC GTG GAA TTT CCT CTG ACA TA -3′
MGSAα -97/-62	5′-	GGG ATC GAT CTG GAA CTC CGG GAA TTT CCC TGG CCC -3′
mGC-4	5′-	... ...C T.. ... AA. ... ... -3′
mGC-4B	5′-	... ...A A.. ... GG. ... ... -3′
mAT-2	5′-	... ... .G. ..C ... . -3′
mAT-B	5′-	... ...C ... ... -3′
MGSAα*Sp1*	5′-	ATA AGC TTC CAC CCT GGG GGC GGG GCC GTC GCC TT -3′
MGSAαmSp1	5′-	... ... TT. ... ... -3′
Sp1(SV40)	5′-	CTG GGC GGA GTT AGG GGC GGG ATG GGC GGA GTT AG -3′

Wild type MGSA/GROα and IL-8 sequences are shown. For each mutated oligonucleotide, nucleotides similar to MGSA/GROα are indicated by dots and the mutant nucleotide sequences are shown. The IL-8 oligonucleotide represents sequences –101 to –63 from the human IL-8 promoter (63). The MGSAα promoter sequences shown are from –97 to –62 (51). Mutations in the MGSA/GROα NF-κB element include mGC-4, mGC-4B, mAT-2 and mAT-B. The Sp1 oligonucleotide represents the SV40 promoter Sp1 enhancer (1). The MGSAαSp1 and mutant MGSAα Sp1 oligonucleotides represent sequences –139 to –110 from MGSA/GROα.

Radiolabeled and competitor DNA

Oligonucleotides were synthesized on a Milligen 7500 DNA synthesizer (Diabetes Research DNA Core, Vanderbilt University). Equal amounts of each oligonucleotide and its complement were annealed in STE (10 mM Tris–HCl, pH 7.8; 1 mM EDTA, pH 8.0; 200 mM NaCl) by boiling the oligonucleotides in a water bath which was slowly cooled to room temperature (~4 h). The oligonucleotides (coding strand) are shown in Table 1. Probes for gel mobility shift analysis were prepared by radiolabeling 100 ng of annealed oligonucleotides with T4 polynucleotide kinase.

Nuclear extracts and DNA binding assay

Nuclear extracts were prepared from Hs294T and RPE cells as described (51). Whole cell extracts from COS cells transfected with either CMV-HMGI or CMV parental vector were collected by vigorously shaking collected cell pellets in 50 mM HEPES, pH 7.0; 250 mM NaCl, 5 mM EDTA, pH 8.0; 0.1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin and 5 μg/ml leupeptin. Recombinant HMGI was produced from the HMGI pET15b vector as previously described (31). The HMGI(Y) binding activity was performed by incubating radiolabeled probes with nuclear extracts in a modified binding reaction buffer as described (10 mM Tris, pH 7.5; 50 mM NaCl; 5% glycerol) (31) and 0.5 μg poly dG-dC:poly dG-dC (Pharmacia). Where indicated, nuclear extracts were heat treated at 52°C for 3 min prior to the binding reaction as described (59). Antibody analysis involved pre-incubating the binding reaction buffer with the antisera for 5 min prior to addition of the nuclear extracts for 45 min at room temperature.

Labeled oligonucleotides were then added and reactions were further incubated for 15 min at room temperature prior to analysis by electrophoresis at 4°C in a non-denaturing 6% polyacrylamide gel in 0.5× TBE (45 mM Tris, pH 8.0; 45 mM boric acid; and 1 mM EDTA, pH 8.0). Gel mobility shift analyses of Sp1 binding activity were performed as described earlier (51). Sp1 and Sp3 antibody analyses were performed by incubating nuclear extracts with appropriate antisera 15 min at room temperature prior to addition of radiolabeled probe. After a 15 min room temperature incubation, samples were analyzed by electrophoresis at room temperature in a 1× TGE (25 mM Tris, pH 8.0, 190 mM glycine and 1 mM EDTA, pH 8.0) 6% polyacrylamide gel.

DNase I footprinting

Footprint analysis was done according to established procedure (60) with the exception that the DNase I reactions were stopped by the addition of 2× proteinase K buffer (10 mM Tris, pH 8.0; 20 mM EDTA; 100 mM NaCl; 0.2% SDS; 0.4 mg/ml proteinase K). The binding buffer used for the footprint reactions was identical to that described for the gel mobility shift assays with 1 mM $CaCl_2$ and 2.5 mM $MgCl_2$. The probe was a 220 bp *Ava*II–*Hin*dIII fragment of the MGSA/GROα promoter in Bluescript SK– (Stratagene). The *Ava*II site was end-labeled using T4 polynucleotide kinase. Each footprint reaction contained 5000 c.p.m. probe, 25 μg poly dI-dC:poly dI-dC (Pharmacia), and 200 μg protein extract in 100 μl binding buffer. For oligonucleotide competition, unlabeled annealed oligonucleotides at 50- or 500-fold excess were pre-incubated with extracts 15 min prior to labeled probe addition. Oligonucleotides included MGSAαSp1, MGSAαmSp1, MGSAα-97/-62 and mGC-4 (see Table 1 for sequences). DNase I treatment was with 0.75 U for 2 min at 25°C. Nuclear extracts used in the footprint reactions were prepared essentially according to the method of Dignam *et al.* (61), except that buffer C contained 0.52 M KCl. Following proteinase K digestion for 2 h at 37°C, the DNA was purified by two extractions with phenol/chloroform in the presence of 0.375 M sodium acetate and 25 μg/ml yeast tRNA (Sigma). The aqueous phase was then precipitated as described (60). Footprint reactions were separated on a 7.5% polyacrylamide, 8 M urea sequencing gel, together with a Maxam–Gilbert G+A sequencing tract to provide orientation.

Antibodies

Sp1, Sp3, C/EBPβ, NF-κB p50, NF-κB p52 and NF-κB p65 antisera were obtained from Santa Cruz. An additional Sp3 antisera was the generous gift of Guntram Suske and was as described (8). HMGI(Y) antisera was the generous gift of Raymond Reeves [designated HMGI(Y)-1] and was as described (62). Additional antisera to recombinant HMGI(Y) [designated HMGI(Y)-2] and the appropriate pre-immune antisera were the generous gifts of Dimitris Thanos and Tom Maniatis and were as described (31).

RESULTS

DNase I footprinting reveals Sp1 and NF-κB enhancer regions are protected

We and others have previously shown that the NF-κB enhancer was essential for constitutive and cytokine induced MGSA/GRO gene transcription (51–53). Sequence analysis of the immediate MGSA/GROα promoter revealed several consensus elements in addition to the NF-κB element (Fig. 1A). In particular, a perfect consensus site was identified for the Sp1 transcription factor. Furthermore, there were weak nucleotide similarities with the NF-IL6 binding element identified in several other immune response genes including IL-8 and IL-6 (63,64). To determine whether these identified regions were recognized by DNA-binding proteins, DNase I footprint experiments were performed using untreated and IL-1 stimulated Hs294T melanoma cell nuclear extracts and a 220 bp *Ava*II–*Hin*dIII restriction fragment encompassing the Sp1 and NF-κB sequences. Footprint analysis resulted in two detectable protected regions (Fig. 1B). Similar results were observed using the identical restriction fragment and nuclear extracts from IL-1 stimulated RPE cells (data not shown). Furthermore, the addition of wild type Sp1 oligonucleotide (Sp1) at 50- or 500-fold excess resulted in a loss of the protected region B while mutant Sp1 oligonucleotide (mSp1) had no affect (Fig. 1B). Likewise, competition with an oligonucleotide encompassing MGSA/GROα –97 to –62 resulted in loss of the protected region A while a similar oligonucleotide with point mutations in the NF-κB region had no effect (Fig. 1B). This indicated that the two regions recognized by nuclear DNA binding proteins were specific for the Sp1 and NF-κB elements.

Sp1 and Sp3 complexes interact with MGSA/GROα enhancer region

Gel mobility shift analyses with RPE nuclear extracts indicated two complexes bound to an oligonucleotide containing the MGSA/GROα Sp1 consensus element (Fig. 2A). Addition of either 50- or 1000-fold excess MGSA/GROα Sp1 oligonucleotide completely eliminated the two complexes (Fig. 2A). We have also observed that 50-fold excess of SV40 early promoter Sp1 oligonucleotide (1) removed the two complexes. Addition of 50- or 1000-fold excess MGSA/GROα Sp1 oligonucleotide with point mutations in the Sp1 consensus element did not affect the binding of the two bound complexes (Fig. 2A). Inclusion of Sp1 antisera in the binding reaction resulted in a partial elimination of the upper complex, while Sp3 antisera eliminated the lower complex and diminished the upper complex (Fig. 2B). An additional Sp3 antisera (8) also removed the lower complex and partially eliminated the upper complex (data not shown). As negative controls, pre-immune and NF-κB p50 antisera were included in the binding reaction; neither had an effect on the bound complexes (Fig. 2B). These results indicated that both Sp1 and Sp3 specifically recognize the MGSA/GROα Sp1 element.

Effect of Sp1 loss on MGSA/GROα promoter activity

Previous deletion analysis of the MGSA/GROα promoter demonstrated that the NF-κB element and immediately adjacent regions located between –100 and –43 in relation to the transcription start site were essential for basal and cytokine induction (51). More detailed deletion analysis of the MGSA/GROα 350 bp promoter region indicated that the region 42 bp upstream of the NF-κB element also significantly contributed to the basal CAT promoter activity (unpublished observations). This region contains the Sp1 enhancer region that is protected in the DNase footprint analysis (Fig. 1B). To determine the role for this Sp1 enhancer region in MGSA/GROα regulation, we created point mutations in the Sp1 consensus element within MGSAα350/CAT. Wild type and mutant Sp1 MGSAα350/CAT were

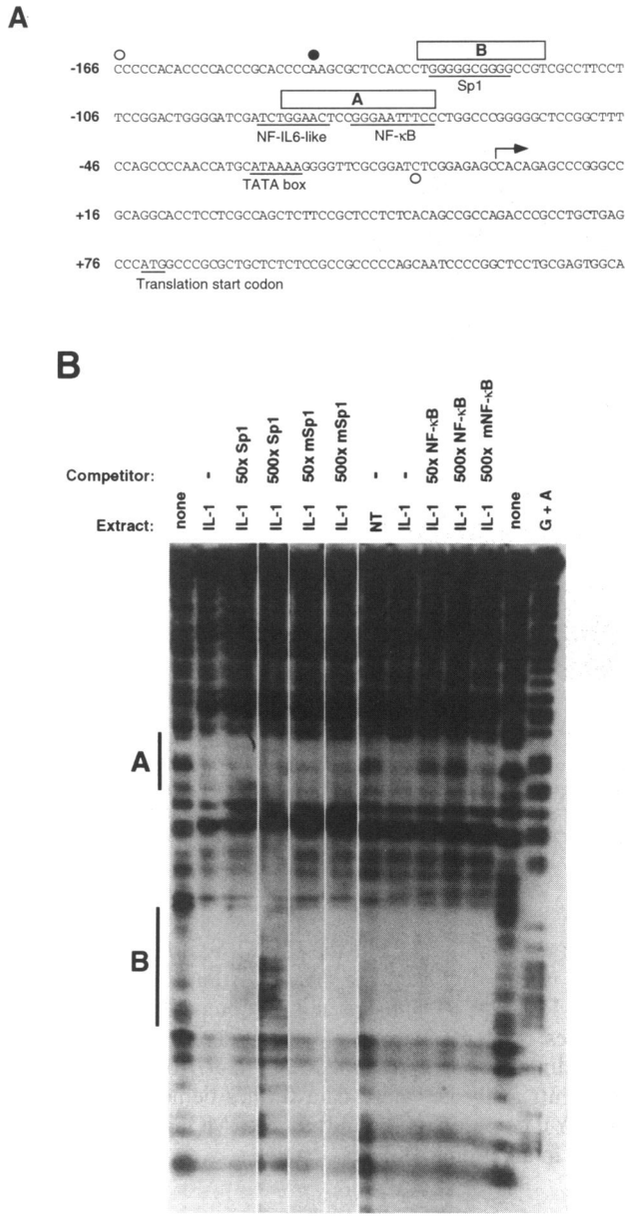

Figure 1. DNase I footprinting of the MGSA/GROα promoter reveals two protected regions. (**A**) Sequence analysis of the MGSA/GROα promoter. Consensus DNA binding elements are underlined and indicated. The arrow represents transcriptional start site. Open circles represent MGSA/GROα promoter region present in the *Ava*II–*Hin*dIII restriction enzyme fragment used in DNase footprint region; closed circle indicates initial nucleotide in Maxam–Gilbert G+A tract shown in accompanying footprint. The translation start codon is indicated. The protected regions A and B identified by DNase I footprint are indicated by the labeled boxes. (**B**) A 220 bp *Ava*II–*Hin*dIII fragment of the MGSA/GROα promoter was incubated with nuclear extracts (200 μg) from either unstimulated (NT) or IL-1 (5 U/ml) stimulated (IL-1) Hs294T cells. After DNase I digestion of the binding reaction with extract or probe alone, the DNA was isolated and subsequently separated on 7.5% sequencing gel. Where indicated, either 50- or 500-fold excess of a 35 bp oligonucleotide containing the MGSA/GROα Sp1 or mutated Sp1 element or a 36 bp oligonucleotide containing the NF-κB or mutated NF-κB element was incubated with the nuclear extracts prior to labeled probe addition (see Materials and Methods for oligonucleotide sequences). A Maxam–Gilbert G+A sequencing tract of the labeled probe was included for orientation. Two protected regions (A and B) present in nuclear extracts as compared to probe alone are indicated.

transiently transfected into either RPE or Hs294T cells. Cells were then left unstimulated or IL-1 stimulated for 24 h prior to collection. Upon mutation of the Sp1 element, there was a substantial decrease in basal MGSA/GROα promoter activity in both the RPE and Hs294T cells (Fig. 3). Upon IL-1 stimulation, a similar fold induction was observed with either the wild type or mutant Sp1 MGSAα350/CAT transfected cells, though the level of CAT activity was lower for the mutant Sp1 construct (Fig. 3). These results suggest that cytokine increased MGSA/GROα transcription was not dependent upon the Sp1 element, though basal MGSA/GROα transcription in normal retinal pigmented cells and the Hs294T melanoma cells did require the Sp1 consensus motif.

Characterization of HMGI(Y) bound to MGSA/GROα NF-κB element

In addition to Sp1, the DNase I footprint and promoter deletion analyses indicated that the downstream region containing the NF-κB element was involved in MGSA/GRO promoter activity (Fig. 1B) (51). We previously determined that NF-κB complexes containing both NF-κB p50 and NF-κB p65 significantly contributed to MGSA/GRO basal and cytokine induction in RPE cells and basal promoter activity in Hs294T cells (51). Close examination revealed that the consensus sequence of the MGSA/GRO genes and the closely related IL-8 gene NF-κB enhancers was identical to the IFN-β NF-κB element (PRDII) from the non-coding strand (31). Previous work demonstrated that viral induction of IFN-β through the PRDII region required both the binding of NF-κB complexes and the structural HMGI(Y) proteins (31). We were interested in determining if HMGI(Y) binds to the MGSA/GROα NF-κB element in addition to the identified NF-κB complexes. Gel mobility shift analyses of nuclear extracts from unstimulated and IL-1 stimulated Hs294T cells were performed using an oligonucleotide containing the MGSA/GROα NF-κB element. The unstimulated extracts revealed multiple DNA-binding complexes which we previously identified as NF-κB (51). IL-1 stimulation resulted in a significant increase in the NF-κB heterodimer complex and the appearance of a fast migrating doublet (Fig. 4). This doublet was identical to that previously seen by others as the HMGI(Y) doublet bound to DNA (29,31). Similarly, IL-1 stimulation of RPE cells increased both the upper NF-κB complexes and lower doublet (data not shown).

Since HMGI(Y) recognizes AT-rich regions, poly dA-dT:poly dA-dT was included as a specific competitor. In addition, HMGI(Y) binding is heat stable at 52°C (59); therefore, we heat treated the nuclear extracts at 52°C for 3 min. Binding reactions with heat treated extracts resulted in a significant loss in binding of the heat labile NF-κB complexes with the lower doublet retained (Fig. 4). The addition of poly dA-dT:poly dA-dT completely eliminated the lower complexes present in both extracts with no effect on the upper NF-κB containing complexes present in IL-1 stimulated Hs294T nuclear extracts (Fig. 4). The heat stability and competition by AT-rich sequences suggested strongly that the lower complexes were HMGI(Y) related.

In contrast to results described in Figure 4, the fast migrating doublet was detectable in cells that have not been stimulated with IL-1 (compare Figs 4 and 5A). The elevated presence of the NF-κB p50/p65 heterodimer suggests that these Hs294T cells were being actively stimulated by secreted factors. Generally,

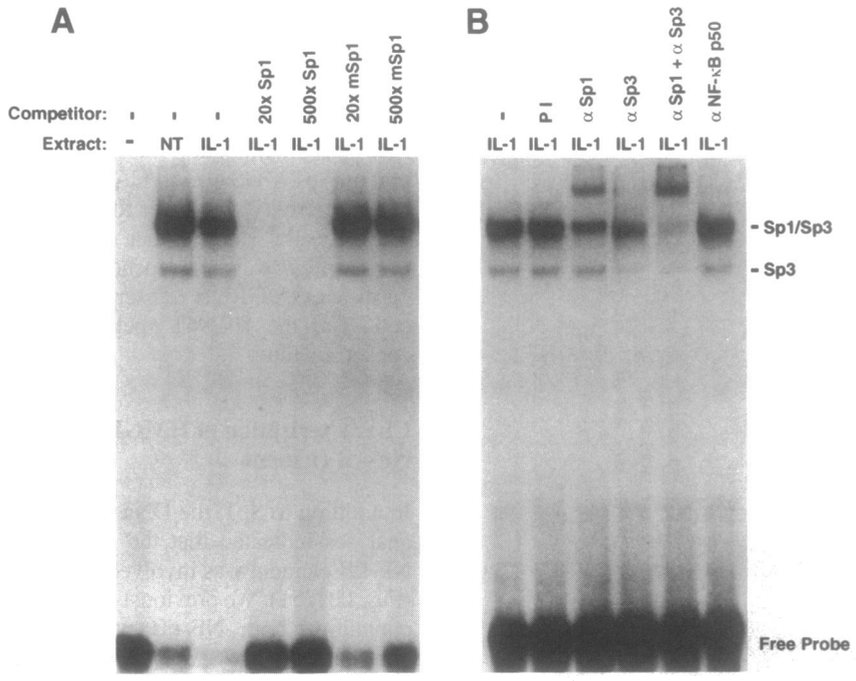

Figure 2. Characterization of MGSA/GROα Sp1 enhancer specific complexes. (**A**) Extracts (5 μg) from either unstimulated (NT) or 5 U/ml IL-1 stimulated (IL-1) RPE cells were incubated with ~20 000 c.p.m. ^{32}P-radiolabeled MGSAαSp1 oligonucleotide probe for 15 min at room temperature. Prior to addition of the indicated probe, unlabeled Sp1 (Sp1) or mutant Sp1 (mSp1) oligonucleotide at either 50- or 1000-fold excess were incubated with the IL-1 nuclear extracts for 15 min at room temperature. (**B**) IL-1 stimulated RPE nuclear extracts (5 μg) were pre-incubated with either pre-immune serum (PI) or antisera to Sp1 (αSp1), Sp3 (αSp3), a combination of both Sp antisera (αSp1+αSp3), or NF-κB p50 (αNF-κB p50) for 15 min prior to labeled probe addition. Resulting protein–DNA complexes were separated on 1× TGE polyacrylamide gels.

RPE cells in culture can be arrested such that there is cytokine induction of the binding of HMGI(Y) and NF-κB complexes.

To confirm the identity of the retarded complexes, we performed gel mobility shift analyses with Hs294T nuclear extracts pre-incubated with various NF-κB and HMGI(Y) antisera. Non-specific controls included pre-immune and C/EBP-β antisera. Poly dA-dT:poly dA-dT oligonucleotides were included as a positive control. As previously demonstrated, NF-κB p50 and NF-κB p65 bound to the MGSA/GROα enhancer region (Fig. 5A) (51), while NF-κB p52 and c-rel were not components of the bound complexes. Two separate anti-HMGI(Y) antisera eliminated the doublet bound to the MGSA/GROα wild type NF-κB element (Fig. 5A and B). The HMGI(Y) antisera developed by Thanos and Maniatis [designated αHMGI(Y)-2] also interfered with the binding of the upper complexes, while the HMGI(Y) antisera developed by Raymond Reeves [designated αHMGI(Y)-1] did not (Fig. 5B). These data demonstrated the presence of HMGI(Y) and NF-κB bound to the MGSA/GROα enhancer region.

To further characterize the HMGI(Y) complexes bound to the MGSA/GROα NF-κB element, we examined the binding of COS cell expressed HMGI to this element. Briefly, COS cells were transiently transfected with a CMV-driven HMGI vector. Immunoblot analysis indicated that COS cells transfected with the CMV parental vector have a low amount of HMGI(Y) that is significantly increased upon transfection of the CMV-HMGI vector (data not shown). Gel shift analyses performed by incubating a radiolabeled MGSAα-97/-62 DNA probe with the COS whole cell extracts indicated a predominant fast migrating complex which migrated like the HMGI(Y) doublet identified in Hs294T nuclear extracts (Fig. 6). This complex was eliminated by both HMGI(Y)-1 antisera and by competition with AT-rich sequences (Fig. 6). We have also observed that recombinant HMGI similarly recognizes the MGSAα-97/-62 oligonucleotide in gel shift analysis. These data further demonstrated that the HMGI(Y) complex is bound to the MGSA/GROα NF-κB element in addition to NF-κB.

HMGI(Y) recognizes AT-rich motif nested within MGSA/GROα NF-κB element

HMGI(Y) binds selectively to stretches of AT-rich regions of DNA; therefore, HMGI(Y) recognizes only those NF-κB elements with AT-rich centers such as previously identified for the IFN-β and E-selectin promoters (31). Likewise, Lewis *et al.* have also demonstrated that HMGI(Y) recognizes the AT-rich NF-κB elements in the E-selectin promoter (33). The MGSA/GRO and IL-8 genes have this identical AT-rich region nested within the NF-κB element (Fig. 7A). Point mutations in MGSA/GROα NF-κB element were made in the outer GC-rich region (mGC-4, mGC-4B) or the inner AT-rich region (mAT-2, mAT-B). The mutation mAT-2 converted the MGSA/GROα NF-κB element to the NF-κB enhancer found in the H-2 class I major histocompatibility gene (65), and the mutant mAT-B sequence created the immunoglobulin κ light chain NF-κB enhancer (66) (Fig. 7A). Gel mobility shift analyses utilizing these mutant and wild type oligonucleotide probes incubated with Hs294T nuclear extracts indicated multiple nuclear factor complexes (Fig. 7B). As

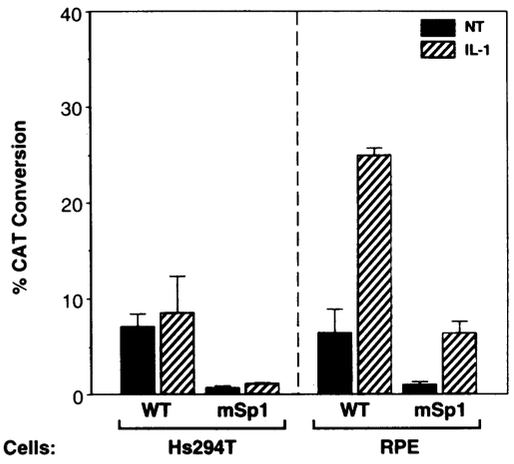

Figure 3. Effect of Sp1 loss on MGSA/GROα promoter activity. Hs294T and RPE cells were transiently transfected with 5 μg of either wild type MGSAα350/CAT (WT) or mutant Sp1 MGSAα350/CAT (mSp1) and 2 μg pCMVhGH as described in Materials and Methods. Approximately 48 h post transfection, cells were either left unstimulated (NT) or stimulated with IL-1 (5 U/ml) (IL-1) for 24 h prior to collection. Transfection efficiencies were normalized by immunodetection of secreted growth hormone. Results are expressed as percent CAT conversion with standard deviations of error from three separate transfections. Results for the Hs294T cell transfection were MGSAα350/CAT wild type control (7.06±1.37), IL-1 (8.54±3.85); mutant Sp1 control (0.63±0.31), IL-1 (1.11±0.13). Results for RPE transfection were MGSAα350/CAT wild type control (7.15±2.90), IL-1 (24.92±0.84); mutant Sp1 control (1.04±0.15), IL-1 (6.39±1.25).

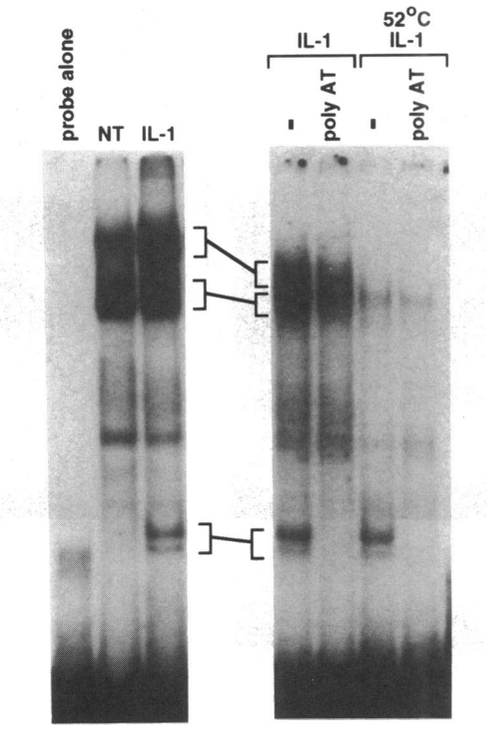

Figure 4. Fast migrating doublet binds to MGSA/GROα NF-κB enhancer region. Nuclear extracts from either unstimulated (NT) or IL-1 (5 U/ml) stimulated (IL-1) Hs294T cells were incubated with 20 000 c.p.m. ^{32}P-radiolabeled MGSAα-97/-62 oligonucleotide. Where indicated, extracts from untreated or 52°C heat treated nuclear extracts were pre-incubated with 50 ng poly dA-dT:poly dA-dT (200-fold excess) for 15 min prior to addition of radiolabeled probe for 20 min at room temperature. Resulting protein–DNA complexes were separated on 0.5× TBE polyacrylamide gels. Similar shifted complexes between the two gels are indicated by joined brackets.

demonstrated previously, both HMGI(Y) and NF-κB were detected bound to wild type MGSAα-97/-62. NF-κB complexes remain bound to mAT-2 and mAT-B probes; however, HMGI(Y) binding was lost. The NF-κB p50/p65 and NF-κB p65 complexes no longer recognized the mGC-4 and mGC-4B oligonucleotide probes; however, HMGI(Y) remained present (Fig. 7B). The residual binding to the mGC-4 and mGC-4B oligonucleotide probes (Fig. 7B) was not NF-κB related since several NF-κB antisera did not eliminate the residual retarded complexes, nor did purified NF-κB p65 recognize these mutant oligonucleotide probes. Separation of the binding reactions on Tris-glycine gels allowed a better resolution of the NF-κB complexes (51) and revealed that the mAT-2 oligonucleotide bound significantly less of the NF-κB p65 homodimer, though the NF-κB p50/p65 heterodimer was retained (data not shown). With these electrophoretic conditions, HMGI(Y) binding was not observed. The loss of the NF-κB p65 homodimer is presumably attributable to the conversion to a weak NF-κB p65 homodimer binding site (67). In summary, analyses of the point mutations created within the MGSA/GROα NF-κB element by gel shift analyses demonstrated that the NF-κB complexes bound to the outer GC-rich region while the HMGI(Y) complexes specifically recognized the inner AT-rich region.

Effect of HMGI(Y) and NF-κB loss on MGSA/GROα promoter activity

Recent work illustrated that loss of either NF-κB or HMGI(Y) binding to the IFN-β PRDII region resulted in loss of viral induction (31). We have previously shown that the NF-κB complex binding was necessary for both basal and cytokine induced expression of MGSA/GROα (51). We were interested in determining whether MGSA/GROα gene regulation required HMGI(Y) binding in addition to the NF-κB complex. Using the NF-κB point mutations demonstrated to hinder either HMGI(Y) or NF-κB complex binding by gel shift analyses, we created mutant NF-κB elements within a CAT reporter construct containing 350 bp of the MGSA/GROα promoter. Point mutations in either the outer GC-rich (mGC-4) or inner AT-rich (mAT-2, mAT-B) NF-κB regions substantially reduced basal MGSA/GROα promoter activity to <30% of wild type promoter upon transfection into Hs294T cells (Fig. 8A). Likewise in RPE cells, a similar decrease in basal expression was observed for mGC-4 and mAT-2; however, mAT-B did not significantly effect basal MGSAα350/CAT expression (Fig. 8B). Mutations of the NF-κB element in either the GC-rich (mGC-4) and AT-rich (mAT-2 and mAT-B) regions significantly diminished IL-1 induction of MGSA/GROα promoter activity (Fig. 8B). These results demonstrated that the MGSA/GROα 350 bp promoter region required both the NF-κB complexes and HMGI(Y) for full cytokine induction.

Thanos *et al.* have demonstrated that HMGI(Y) enhanced NF-κB p50/p65 heterodimer binding to the IFN-β enhancer region *in vitro*. Furthermore, reduction of HMGI(Y) protein levels by antisense expression did not allow NF-κB p50/p65 heterodimer transactivation of the IFN-β promoter in transient

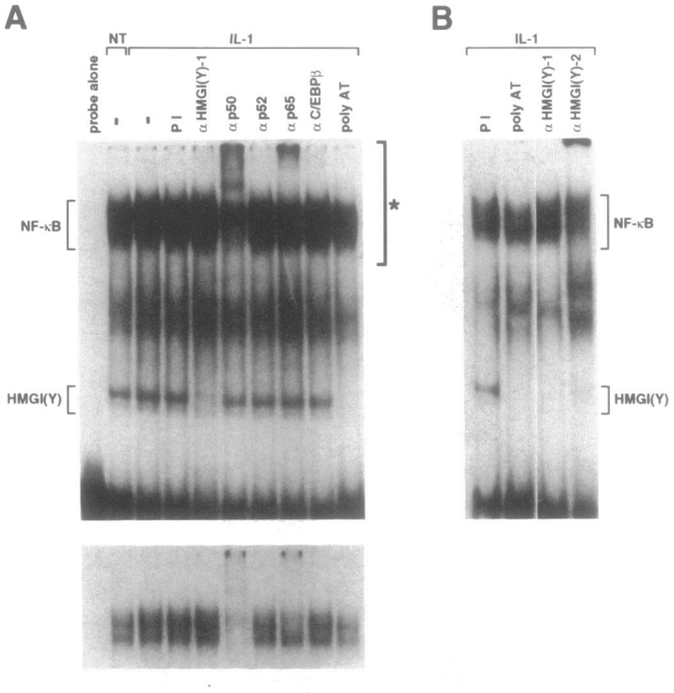

Figure 5. NF-κB and HMGI(Y) bind simultaneously to MGSA/GROα NF-κB enhancer region. (**A**) Nuclear extracts from either unstimulated (NT) or IL-1 stimulated (IL-1) Hs294T cells were incubated with radiolabeled MGSA/GROα-97/-62 oligonucleotide probe. Prior to probe addition, nuclear extracts were incubated with either pre-immune antisera (PI), the indicated antisera, or poly dA-dT:poly dA-dT as described in Materials and Methods. The lower panel is a lesser autoradiograph exposure of the indicated upper region of the gel shift (*). (**B**) Prior to probe addition, nuclear extracts were incubated with either pre-immune (PI), HMGI(Y)-1 or HMGI(Y)-2 antisera. The resulting protein–DNA complexes were separated on 0.5× TBE polyacrylamide gels. The NF-κB complexes are designated, as is the faster migrating HMGI(Y) doublet near the free probe.

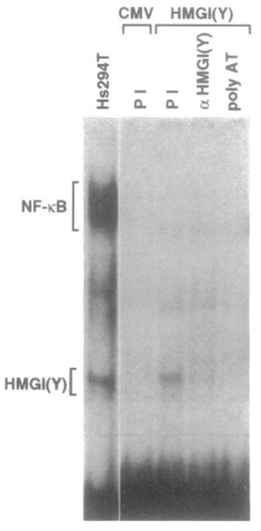

Figure 6. COS cell expressed HMGI binds MGSAα-97/-62 region. Whole cell extracts from COS cells transiently transfected with either the parental CMV vector or HMGI expression vector were pre-incubated with either pre-immune antisera (PI), HMGI(Y)-1 antisera or poly dA-dT:poly dA-dT prior to addition of radiolabeled MGSAα-97/-62 oligonucleotide. For comparison, crude Hs294T IL-1 stimulated nuclear extracts were incubated with the radiolabeled probe. The resulting protein–DNA complexes were separated on 0.5× TBE polyacrylamide gels.

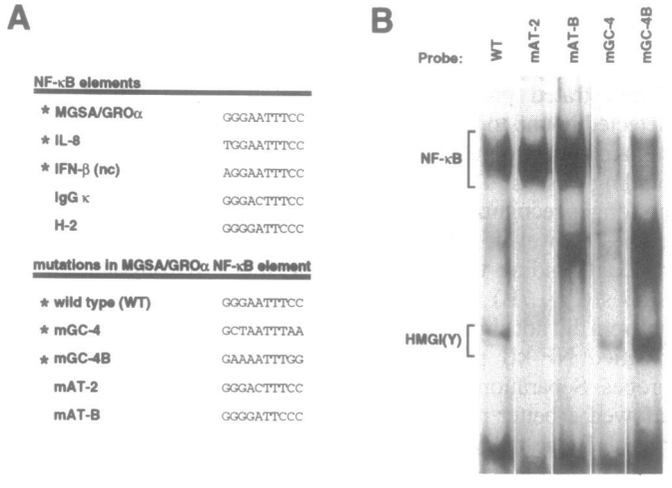

Figure 7. NF-κB and HMGI(Y) bind to distinct nucleotides in MGSA/GROα NF-κB enhancer. (**A**) Sequence similarities between MGSA/GROα, IL-8 (63), IFN-β (31,74), immunoglobulin κ light chain (IgG κ) (66,75), and H-2 class I major histocompatibility (H-2) (65) genes are shown. The IFN-β element is from the noncoding strand. Point mutations made within the MGSA/GROα NF-κB outer GC-rich (mGC-4, mGC-4B) or inner AT-rich (mAT-2, mAT-B) regions are also included. NF-κB elements with AT-rich nested regions (5 nucleotides or more) are denoted with an asterisk. (**B**) Extracts (5 μg) from IL-1 stimulated (IL-1) Hs294T cells were incubated with 20 000 c.p.m. ^{32}P-radiolabeled MGSA/GROα oligonucleotide probes that had either the endogenous NF-κB element or mutations in either the inner AT-rich region (mAT-2 and mAT-B) or the outer GC-rich region (mGC-4 and mGC-4B) (see Table 1) for 20 min at room temperature. Resulting protein–DNA complexes were separated on 0.5× TBE polyacrylamide gels. NF-κB complexes are designated, as is the faster migrating HMGI(Y) doublet near the free probe.

transfection assays (31). To determine whether HMGI(Y) has such a role with MGSA/GROα activation, we co-transfected various MGSAα350/CAT constructs with point mutations in the NF-κB element with an NF-κB p65 (RelA) expression vector. Basal expression was reduced >50% for each of constructs when the promoter region had point mutations in the outer GC-rich region or inner AT-rich region of the NF-κB element (Fig. 9). NF-κB p65 co-transfection significantly increased wild type MGSAα350/CAT activity. NF-κB p65 transactivated through the various mutants, although to a lesser extent (Fig. 9). These results indicated that nucleotides within both the outer and inner region of the MGSA/GROα NF-κB element were necessary for NF-κB p65 transactivation.

DISCUSSION

Sp1 is necessary for basal MGSA/GROα transcription

Several transcription factor binding elements in the MGSA/GROα promoter have been identified in the 260 bp promoter region upstream of the TATA box. We have previously demonstrated that this region is sufficient for IL-1 and TNFα induction in normal RPE cells (51). Furthermore, the NF-κB element located in this promoter region has a significant role in both the basal and cytokine induced MGSA/GROα transcription in Hs294T and RPE cells, respectively (51). DNase I footprinting reveals two regions protected within this promoter region in IL-1 stimulated Hs294T extracts. One of the regions contains the

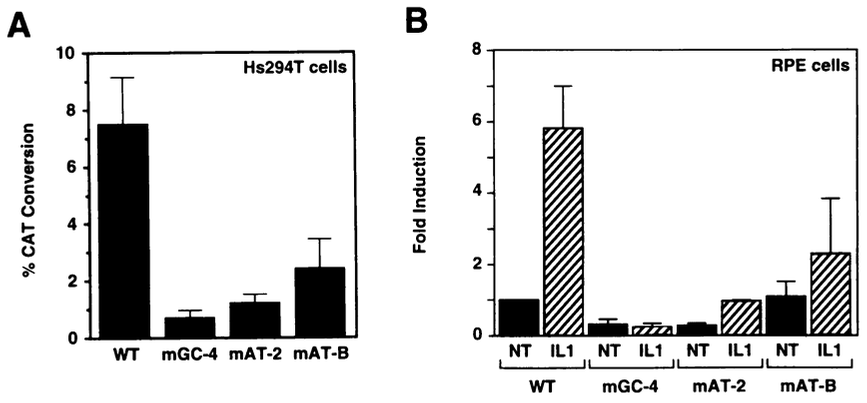

Figure 8. Effect of HMGI(Y) and NF-κB loss on MGSA/GROα promoter activity in RPE and Hs294T cells. Either Hs294T (**A**) or RPE (**B**) cells were transiently transfected with 10 μg of the indicated MGSAα350/CAT construct and 2 μg pCMVhGH as described in Materials and Methods. Approximately 48 h post transfection, cells were either left unstimulated (NT) or IL-1 (5 U/ml) stimulated (IL-1) for 24 h prior to collection. Transfection efficiencies were normalized by immunodetection of secreted growth hormone. Results from three separate Hs294T (**A**) transfections are expressed as percent CAT conversion with standard deviation of error: MGSAα350/CAT WT control (7.47±1.68), mGC-4 (0.70±0.28), mAT-2 (1.20±0.32), and mAT-B (2.42±1.04). Results for RPE (**B**) transfections are expressed as fold activation over cells transfected with wild type MGSAα350/CAT alone with no treatment. Fold induction and range of error for duplicates from two separate RPE transfections were MGSAα350/CAT WT control (1.00) IL-1 (5.82±1.21), mGC-4 control (0.32±0.16) IL-1 (0.24±0.11), mAT-2 control (0.28±0.07) IL-1 (0.95±0.04) and mAT-B control (1.07±0.47) IL-1 (2.28±1.59).

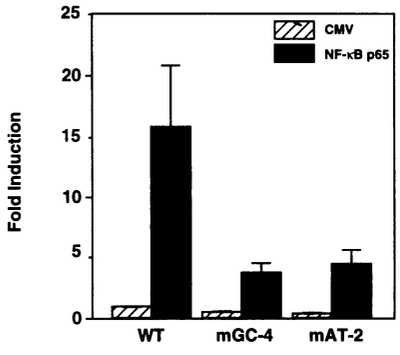

Figure 9. Activation of MGSA/GROα NF-κB mutants by NF-κB p65. RPE cells were co-transfected with 5 μg of the indicated MGSAα350/CAT constructs, 5 μg CMV NF-κB p65 and 2 μg pCMVhGH. The parental CMV expression vector was used to normalize the DNA concentration of all samples. Transfection efficiencies were normalized by immunodetection of secreted growth hormone. Cells were collected 48 h post transfection with one medium change. CAT activity was determined as described in Materials and Methods. Results are expressed as fold induction over cells transfected with wild type MGSAα350/CAT alone. Fold induction and standard deviation of error for three separate transfections were: MGSAα350/CAT wild type control (1.00), NF-κB p65 (15.76±5.10); mGC-4 control (0.43±0.13), NF-κB p65 (3.77±0.85); mAT-2 control (0.33±0.12), NF-κB p65 (4.48±1.24).

NF-κB element while the other contains the Sp1 consensus element located 42 bp upstream of the NF-κB element. Gel shift analyses of nuclear extracts from unstimulated or cytokine stimulated RPE and Hs294T cells demonstrate Sp1 and Sp3 complexes bind specifically to the MGSA/GROα Sp1 element.

Sp1 and NF-κB complexes have been reported to physically interact *in vitro* (9,10). HIV-1 LTR promoter activity in both *in vitro* or Jurkat cells requires both the NF-κB and Sp1 motifs, since loss of either element significantly reduces induction of promoter activity (68,69). Likewise, mutation of the Sp1 consensus element in the GM-CSF promoter inhibits NF-κB transactivation, though the NF-κB element is still present (70). Furthermore, the human MCP-1 promoter requires the Sp1 and NF-κB elements for basal and cytokine induced activity, respectively (71). When the MGSA/GROα Sp1 element is either deleted or mutated within MGSA/GROα promoter constructs, we observe a significant decrease in basal promoter activity (>50% decrease in CAT activity). The decrease in CAT activity is similar to that observed with mutation of the NF-κB element [(51) and Fig. 8]. The Sp1 element is not necessary for cytokine induction of MGSA/GROα in RPE cells since the equivalent IL-1 induction of mutant Sp1 MGSAα350/CAT as compared to wild type is observed, though the level of CAT activity is much lower. The lower level is presumably due to the marked decrease in basal CAT activity. However, these results strongly suggest that the Sp1 consensus element has a significant role in basal MGSA/GROα expression in both RPE and Hs294T cells.

The presence of Sp3 in nuclear extracts of Hs294T and RPE cells is intriguing. Sp3 has been implicated to be a repressor of Sp1 since Sp3 lacks a transactivation domain and competes with Sp1 for binding (5,7,8). Alternatively, Sp3, like Sp1, can also serve as an activator (6,15). These differences in activity may be due to the presence of co-activators such as Rb or other transcription factors that allow Sp3 to serve as an activator of certain genes. We are not able at this time to distinguish whether Sp1, Sp3 or both contribute to the constitutive MGSA/GRO promoter activity. However, point mutations in the GC-rich Sp1 element result in loss of both Sp1 and Sp3 binding and significantly diminish basal MGSA/GROα promoter activity.

HMGI(Y) in addition to NF-κB is required for MGSA/GROα promoter activity

We have determined that the NF-κB enhancer sequence for the chemokines MGSA/GRO and IL-8 are identical to that of IFN-β in that there is an AT-rich region nested within the NF-κB consensus element. Furthermore, the MCP-1 NF-κB element is identical to the MGSA/GRO NF-κB element (71). The E-selectin gene has also been demonstrated to contain two NF-κB elements

with AT-rich regions (32,33). To date, these are the only inflammatory genes identified which contain NF-κB elements with AT-rich centers.

We demonstrate here that the MGSA/GROα NF-κB enhancer binds both HMGI(Y) and NF-κB and that these complexes are important for transactivation through this promoter element. Previous work on the IFN-β and E-selectin promoters indicated that the presence of HMGI(Y) enhances NF-κB binding to its recognition sequences putatively through direct protein–protein interactions (31,33). While that work utilized recombinant NF-κB and HMGI(Y) proteins to observe binding to the NF-κB enhancers, we demonstrate here from melanoma cell nuclear extracts that endogenous NF-κB and HMGI(Y) bind to the MGSA/GROα NF-κB element. Two different HMGI(Y) antisera eliminated the HMGI(Y) doublet that migrates near the free probe by gel shift analyses. However, only one of the HMGI(Y) antisera [HMGI(Y)-2 developed by Thanos and Maniatis] affects the formation of the upper NF-κB complexes, suggesting a possible interaction between HMGI(Y) and NF-κB. One possible explanation for the difficulty in observing an HMGI(Y) and NF-κB ternary complex by gel shift analyses is the antigenic sites may not be available for antibody recognition due to conformational restraints when the ternary complex binds to the DNA.

Interestingly, by gel shift analysis, we have observed variability in the level of HMGI(Y) present in non-cytokine treated Hs294T melanoma cells (compare Figs 4 and 5A). Based on prior reports demonstrating HMGI(Y) is expressed at high levels in transformed and rapidly growing cells (26–28), it is possible that variability in HMGI(Y) levels is due to differences in endogenous cytokine production or proliferation of the cultured cells. Since Hs294T melanoma cells produce growth factors and cytokines such as IL-1 and thus resist density arrest, it is likely that there would be associated changes in nuclear transcription factors such as HMGI(Y) and NF-κB.

Point mutations in the AT-rich region of the NF-κB element result in the loss of HMGI(Y) binding and a significant decrease in basal promoter activity in both the Hs294T melanoma cells and normal retinal pigment epithelial (RPE) cells. Cytokine induction of MGSA/GROα is significantly diminished in RPE cells with loss of HMGI(Y) binding to the MGSA/GROα NF-κB element. These studies are in agreement with IFN-β and E-selectin promoter mutagenesis studies in that conversion of their NF-κB enhancers to variant NF-κB enhancers that do not bind HMGI(Y) results in a significant loss of viral and cytokine induction, respectively (31–33).

In addition to the loss of HMGI(Y) binding, the subsequent decrease in MGSA/GROα basal and cytokine induced promoter activity upon mutation of the NF-κB element to the H-2 NF-κB enhancer (mAT-2) may also be correlated with loss of the NF-κB p65 homodimer binding. However, a second set of point mutations in the AT-rich region (mAT-B) retains both NF-κB p65 homodimer and NF-κB p50/p65 heterodimer binding and loses HMGI(Y) binding. This mutant mAT-B exhibits diminished basal promoter activity in Hs294T cells and diminished cytokine induction in RPE cells. Our results agree with those obtained with E-selectin in that loss of HMGI(Y) partially reduces cytokine induction (32,33).

In summary, our data provide evidence for the requirement for several different classes of DNA-binding proteins for the regulation of the MGSA/GROα promoter. Based on previous studies that suggest physical interactions (cross-talk) between such factors may be important for their combined action, further analysis of these interactions is suggested. In particular, though we have shown here that both the Sp1 and NF-κB enhancers are required for basal MGSA/GROα transcriptional activity, it remains to be determined if these two regions cooperate such that a ternary complex with DNA is formed to regulate MGSA/GROα gene expression. Recent evidence now supports an interaction between transcription factors implicated in DNA bending such as Sp1 and HMGI(Y) with other transcription factors including NF-κB (9,10,30,31,72). Interestingly, both the NF-κB and Sp1 elements found in the M-CSF promoter are required for the elevated basal transcription of M-CSF within K562 leukemic cells (73). Like MGSA/GROα expression in the Hs294T melanoma cells as compared to non-transformed pigmented cells (51), M-CSF expression in leukemic cells is primarily regulated post-transcriptionally by PMA and cycloheximide, though the basal transcription rate is elevated compared to other hematopoeitic cell lines (73). Moreover, HMGI(Y) has been implicated in the constitutive expression of lymphotoxin (TNFβ) in several leukemic cell lines (29). It will be interesting to determine if the Sp1, C/EBP-like and NF-κB/HMGI(Y) elements interplay for specificity in MGSA/GROα transcriptional regulation in normal and transformed cells. Such an interaction will add to the idea that cells may utilize combinatorial effects to control the expression of a multitude of genes with similar transcription factor binding regions.

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