Constitutive and Cytokine-induced Expression of the Melanoma Growth Stimulatory Activity/GROα Gene Requires Both NF-κB and Novel Constitutive Factors*

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Chemokines play a major role in mediation of inflammation and wound healing. Melanoma growth stimulatory activity (MGSA/GRO) and interleukin-8 (IL-8) are highly related chemokines that have a causal role in melanoma progression. Expression of these chemokines is similar in that both require the NF-κB element and additional regions such as the CAAT/enhancer binding protein (C/EBP) element of the IL-8 promoter. The constitutive and cytokine IL-1-induced promoter activity of the chemokine MGSA/GROα in normal retinal pigment epithelial and the Hs294T melanoma cells is partially regulated through the NF-κB element, which binds both NF-κB p50 and RelA (NF-κB p65) homodimers and heterodimers. Mutational analysis of the MGSA/GROα promoter reveals that, in addition to the NF-κB element, the immediate upstream region (IUR) is necessary for basal expression in retinal pigment epithelial and Hs294T cells. Gel mobility shift and UV cross-linking analyses demonstrate that several constitutive DNA binding proteins interact with the IUR. Although this region has sequence similarity to the several transcription factor elements including C/EBP, the IUR includes sequences that have no similarity to previously identified enhancer regions. Furthermore, RelA transactivates through either the NF-κB element or the IUR, suggesting a putative interaction between NF-κB and this novel complex.

Chemokines play a major role in mediation of inflammation and wound healing. Melanoma growth stimulatory activity (MGSA/GRO) and IL-8, members of the C-X-C chemokine family, are similar at both the structural and functional level (chemokines reviewed in Ref. 1). These proteins share several common receptors including the IL-8 A and B receptors and the Duffy antigen receptor for chemokines (DARC) (2–7). Both MGSA/GRO and IL-8 are highly chemotactic for neutrophils (8–12). Northern analyses and immunochemical studies have revealed that MGSA/GRO and/or IL-8 are produced by several cell types including endothelial cells (13, 14), fibroblasts (8, 15–17), keratinocytes (8, 18–21), and retinal pigment epithelial cells (22).

MGSA/GRO expression increases as melanocytes progress to malignant melanoma (18, 23, 24). Furthermore, both MGSA/GRO and IL-8 serve as autocrine growth factors for several melanoma cell lines (9, 18, 25–27). Three MGSA/GRO genes have been identified (MGSA/GROα, -β, and -γ) (28–31). The MGSA/GROα form was purified from culture medium conditioned by Hs294T human melanoma cells (16, 32, 33), and overexpression of MGSA/GROα in immortalized mouse melanocytes enabled these cells to form tumors in athymic nu/nu mice (34). We have shown in Hs294T cells that there is a high constitutive level of transcription of the MGSA/GROα gene and that can not be significantly induced by the cytokines IL-1 and tumor necrosis factor α. In contrast, IL-1 markedly increases MGSA/GROα gene transcription in normal retinal pigment epithelial (RPE) cells (35).

The human IL-8 and MGSA/GRO genes contain an NF-κB element within their enhancer regions that has been shown to be necessary for transcriptional activation of these chemokines (35–37). Furthermore, IL-8 gene regulation also requires the C/EBP element adjacent to the NF-κB element (36). The C/EBP and NF-κB complexes directly interact or cross-couple to further enhance IL-8 gene transcription (38–40). Although the chemokine MGSA/GRO has an essential role in inflammation and tumor progression, regulation of MGSA/GRO gene expression is not as well understood as the closely related IL-8 gene.

In this work, we demonstrate that in addition to the NF-κB element, the immediate upstream region (IUR) is necessary for basal and cytokine induced expression of MGSA/GROα in RPE cells. Likewise, basal MGSA/GROα promoter activity within the Hs294T melanoma cells also requires this region. However, unlike the IL-8 promoter, neither C/EBPα nor C/EBPβ recognize the similar region in the MGSA/GROα promoter. Furthermore, RelA (NF-κB p65) transactivates MGSA/GROα transcription either directly through the NF-κB element or indirectly through the adjacent IUR. We have identified a novel complex that is constitutively bound to the IUR in both normal RPE and melanoma cells. We propose that transcriptional regulation of the MGSA/GROα gene involves multiple factors that recognize the NF-κB and immediate surrounding regions.

MATERIALS AND METHODS

Northern Blot Analysis—Hs294T and RPE cells were cultured as described by Shattuck et al. (35). Total RNA was purified as described previously (35). Random primed 700-base pair EcoRI fragment of MGSA/GROα cDNA (16) and a 400-base pair EcoRI fragment of IL-8 cDNA (41) were used as probes. Hybridization of cyclophilin (1B15) was used as a standard for quantitation.
The NF-IL-6 oligonucleotide represents -168 to -137 from IL-6 promoter, which contains the NF-IL-6 element (43). Wild-type IL-8 and MGSα/GROα sequences are shown. For each mutated oligonucleotide, nucleotides similar to MGSA/GROα are indicated by dots, and mutant nucleotides are shown. Mutations in the MGSA/GROα NF-κB element include mNF-κB and mIUR + mxB. Mutations in the MGSA/GROα IUR include mIUR, mIUR-B, and mIUR-C. Wild-type MGSA 2x IUR and respective mutant (MT 1-4) oligonucleotides contain two repeats of the underlined region in MGSA/GROα -97/-62.

CAT Reporter Gene Plasmid Construction—MGSAα350/CAT and MT 1

NF-IL6
IL-8 -101/-63
MGSαα -97/-62
mIUR
mIUR-B
mIUR-C
mIUR + mxB
MGSαα -97/-78
mIUR
MGSαα -97/-78
MGSαα 2x IUR
MT 1
MT 2
MT 3
MT 4

NF-IL6
IL-8 -101/-63
MGSαα -97/-62
mIUR
mIUR-B
mIUR-C
mIUR + mxB
MGSαα -97/-78
mIUR
MGSαα -97/-78
MGSαα 2x IUR
MT 1
MT 2
MT 3
MT 4

Expression Vector Constructs and Recombinant Protein—The RelA expression vector was as described previously (42) and was the gift of Warner Greene (University of California, San Francisco). NF-IL6 DNA was as described previously (43) and was the gift of Tadamitsu Kishimoto (Osaka University, Osaka, Japan). Recombinant C/EBPβ and NF-IL6 were produced utilizing pSET vectors as described previously (44) and were the generous gifts of Linda Sealy (Vanderbilt University) and Thomas Waldmann (University of Freiburg, Freiburg, Germany).

Transfection and CAT Reporter Assay—Either RPE or Hs294T cells were co-transfected with 10 μg of the indicated MGSAα/CAT fusion genes (for point mutations see Table I) and 2 μg of pCMVhGH (obtained from Dr. Lynn Matriscian, Vanderbilt University), which allowed for normalization of transfection efficiency by measuring growth hormone secretion. The MGSAα/CAT reporter plasmids were obtained from Linda Sealy (Vanderbilt University) and Thomas Waldmann (University of Freiburg, Freiburg, Germany). Transfections were performed by the calcium phosphate coprecipitation method (45). CAT enzymatic activity was assayed as described previously (46). The percent 14C]chloramphenicol converted to acetylated forms was determined from phosphoimager analysis (Molecular Dynamics).

Radioactively labeled 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, pH 7.8, 1 mM EDTA, pH 8.0, 200 mM NaCl) by boiling the oligonucleotides in a water bath that was slowly cooled to room temperature (approximately 4 h).

Oligonucleotides (coding strand) are shown in Table I. Probes for gel mobility shift assays were prepared by radioiodolabelling 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 previously (35) with the exception that cell lysis was performed by vortexing vigorously in the presence of buffer A with 1% Nonidet P-40, 0.4 M NaCl, 0.4% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml PMSF, 1 mM dithioretil, and 1 mM diisopropylfluorophosphosphate (22) for 20 min at 37°C prior to probe addition (40,000 cpm/reaction) for 20 min at room temperature. The resulting protein-DNA complexes were separated on a 6% nondenaturing gel as described previously (47). Whole cell extracts were prepared from Jurkat T-cell transfectants by high salt extraction as described previously (47). Gel shift assays were performed as above with 10 μg of Jurkat extract.

UV CROSS-LINKING—Nuclear extract (5 μg) incubated with the labeled MGSAα 2x oligonucleotide (20 μl total reaction volume) was exposed to short wave ultraviolet irradiation for 15 min (Stratalinker). Half of the reaction (10 μl) was separated on a 6% nondenaturing gel for electrophoresis as described above for the DNA binding assay. The remaining 10 μl was heated (95°C) for 5 min in SDS loading buffer (50 mM Tris, pH 6.8, 2% SDS, 10 mM glycerol, 1% β-mercaptoethanol, 0.1% bromphenol blue). Labeled proteins were then separated by electrophoresis on a 5% SDS-polyacrylamide gel and compared with molecular weight standards.

ANTIBODIES—Antibodies to NF-κB p50, RelA, and p50 immunoreactive antisera (42, 48) were the kind gift of Warner Greene. In addition, antisera to RelA, NF-κB p50, NF-κB p52, and C/EBPβ were obtained from Santa Cruz. Additional antibodies directed against C/EBPα, C/EBPβ, and C/EBPγ were the generous gift of Stephen McKnight and were as described previously (49). Anti-leader binding protein (LBP) antisera was generously provided by Robert Roeder (50).

RESULTS

Regulation of MGSAα/GROα and IL-8 mRNA Synthesis by IL-1—MGSAα/GROα and IL-8 mRNA levels increased rapidly in normal RPE cells stimulated with IL-1 (Fig. 1 and Ref. 35). This induction of MGSAα/GROα was primarily due to increased transcription (35). The Hs294T melanoma cells have a constitutive level of both MGSAα/GROα and IL-8 gene expression (Fig. 1). The basal level of activity of both MGSAα/GROα and IL-8 in the Hs294T cells was equal to or greater than the IL-1-induced level in the RPE cells. We had previously demonstrated by promoter deletion studies that the MGSAα/GROα region between -100 and -43 from the transcription start site were necessary for basal expression in Hs294T melanoma cells and basal and cytokine-induced expression in RPE cells (35). Further analysis demonstrated that the NF-κB element within this region was necessary for this activation.

Mukaida et al. (36) had shown that both the NF-κB and the adjacent C/EBP binding elements were required for IL-1 and tumor necrosis factor α activation of IL-8 in a fibrosarcoma cell line. Further work demonstrated that C/EBP proteins bound to the IL-8 promoter and that NF-κB directly interacted with the

TABLE I

Sequences of oligonucleotides used in gel mobility shift assays

| Sequence | Description |
|----------|-------------|
| NF-IL6   | -101/-63    |
| IL-8     | -97/-62     |
| mIUR     | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| mIUR-B   | -5' - CCC GAT CAT TGG CAC ATT GGA TAA TCT CTC GTA TA -3' |
| mIUR-C   | -5' - CGG ATC GAT CTG GAA CTC CGG GAA TTT CCC TGG CCC -3' |
| mIUR + mxB | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MGSαα -97/-78 | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MGSαα 2x IUR | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MT 1     | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MT 2     | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MT 3     | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
| MT 4     | -5' - CCG TGG CAT CAC ATT GCA CAA TCT TAA TAA GC -3' |
C/EBP complexes (38, 39). Sequence analysis between the IL-8 and MGSA/GRO<sub>α</sub> promoter region indicated an almost identical NF-κB element and adjacent C/EBP-like region (Fig. 2). The MGSA/GRO<sub>α</sub> nucleotide sequence adjacent to the NF-κB element contains several nucleotides that are conserved between previously identified C/EBP enhancers and are essential for C/EBP binding and subsequent transactivation for the IL-8, IL-6, albumin gene (DE1), and serum amyloid A genes (36, 43, 51, 52). The MGSA/GRO region also has close homology with the human immunodeficiency virus type I (HIV-1) LBP-1 binding site, which is necessary in addition to the NF-κB and Sp1 for full transcriptional activation of the HIV-1 long terminal repeat (53–56) (Fig. 2). This sequence was of interest since we have recently demonstrated that basal MGSA/GRO<sub>α</sub> promoter activity required both NF-κB- and Sp1-related complexes bound within the immediate promoter (57).

Immediate Upstream Region Is Required for MGSA/ GRO<sub>α</sub> Promoter Activity—To determine if the IUR located adjacent to the NF-κB element was involved in MGSA/GRO<sub>α</sub> regulation as demonstrated for IL-8 gene regulation (36, 38, 58), we studied the activities of MGSA<sub>α</sub>350/ CAT constructs with point mutations in both the NF-κB and upstream region in transiently transfected RPE cells. Mutations in either site resulted in a substantial loss of basal CAT activity (50%) and subsequent loss of IL-1 induction in RPE cells (Fig. 3A). Mutations made in both regions resulted in a complete loss of CAT activity comparable with the activity obtained from the parental vector pPLFCAT, indicating a complete loss of activation through the MGSA/GRO<sub>α</sub> promoter region (Fig. 3A). Likewise, within the Hs294T melanoma cell line, mutation of either the NF-κB or the IUR resulted in a substantial loss in the baseline CAT activity (Fig. 3B). These results indicated that, in addition to the consensus NF-κB element, the IUR element plays an important role in MGSA/GRO<sub>α</sub> gene regulation in both RPE and Hs294T cells.

Characterization of the Nuclear Proteins That Bind to the MGSA/ GRO<sub>α</sub>-97/62 Region— Gel mobility shift analyses were performed to characterize the nuclear factors that bind to the IUR and NF-κB region. Nuclear extracts from RPE cells were incubated with either the wild type (WT), mutant IUR (m.IUR), mutant NF-κB (m.NF-κB) or double mutant (m.IUR + m.NF-κB) MGSA<sub>α</sub>–97/62 probe (Fig. 4). IL-1 induction resulted in the appearance of two shifted nuclear complexes bound to the wild-type MGSA<sub>α</sub>–97/62 oligonucleotide (lanes 1 and 2). Addition of various NF-κB antisera indicated that the lower complex consisted of the NF-κB p50/RelA heterodimer, while the upper complex contained RelA (presumably homodimers) (lanes 4 and 5). Addition of preimmune NF-κB p52 and C/EBPβ antisera had no effect on the shifted complexes (lanes 3, 5, and 7). Gel mobility shift analyses with labeled mutant IUR (m.IUR) MGSA<sub>α</sub>–97/62 oligonucleotide demonstrated the identical pattern of shifted complexes as wild-type oligonucleotide (lanes 8 and 9). Labelled mutant NF-κB (m.NF-κB) or double mutant (m.IUR + m.NF-κB) MGSA<sub>α</sub>–97/62 oligonucleotides did not retard nuclear complexes (lanes 10–13). These data demonstrated that the nuclear complexes bound to the MGSA/GRO<sub>α</sub>–97/62 region are specific for the NF-κB element and not the IUR.

Characterization of MGSA/ GRO<sub>α</sub> C/EBP-like Sequence as a Potential C/EBP Binding Element—Several groups have demonstrated cooperative binding of C/EBP family members with RelA to the IL-8 C/EBP enhancer region (38, 39). However, IL-8 has a weak binding affinity for C/EBP proteins. Using the IL-8 or MGSA/GRO<sub>α</sub> C/EBP-like regions as probes, we have not been able to identify a C/EBP-related complex from RPE or Hs294T nuclear extracts, although C/EBP proteins were present in these extracts based on immunoblot and gel shift analysis with the NF-IL6 consensus oligonucleotide as a probe (data not shown). Purified C/EBPβ or NF-IL6 did not bind to the C/EBP-like region in the MGSA/GRO<sub>α</sub> promoter, although it did bind weakly to the IL-8 C/EBP enhancer region (data not shown). We addressed the possibility that the affinity of C/EBPβ for the MGSA/GRO<sub>α</sub> enhancer region was too weak to detect an interaction after electrophoresis during gel shift analysis. A converse approach was to analyze the ability of the IL-8 and MGSA/GRO<sub>α</sub> regions to compete for C/EBPβ protein bound to the labeled NF-IL6 consensus element (Fig. 5). Unlabeled NF-IL6 oligonucleotide at 5-fold excess to radiolabeled IL-16 probe reduced C/EBPβ binding by >90% (Fig. 5). With 50-fold excess NF-IL6 oligonucleotide, the C/EBPβ complex was completely removed. Unlabeled IL-8–101/–63 oligonucleotide reduced C/EBPβ binding to the NF-IL6 oligonucleotide by >70% at 200-fold excess, while addition of higher concentrations of this oligonucleotide completely removed C/EBPβ binding. In contrast, addition of unlabeled MGSA<sub>α</sub>–97/62 did not reduce C/EBPβ binding even at 2000-fold excess (Fig. 5C). Our data agreed with earlier studies in which purified C/EBPβ

**Fig. 1.** Expression of MGSA/GRO and IL-8 mRNA in the Hs294T melanoma and RPE cells. Total RNA from unstimulated or 5 units/ml IL-1-stimulated RPE or Hs294T cells for the time (hours) indicated was analyzed by Northern blot as described under "Materials and Methods." Identical blots were hybridized with specific cDNA probes for MGSA/GRO and IL-8. Equal loading was verified by subsequently hybridizing the blots with a cDNA probe for the constitutive mRNA cyclophilin (1B15).

**Fig. 2.** Sequence comparison of MGSA/GRO<sub>α</sub>–97/62 and IL-8–100/65 with several consensus transcription factor elements. Sequence comparison of MGSA/GRO<sub>α</sub>–97/62 and IL-8–100/65 with several consensus transcription factor elements. The nucleotide sequences of the MGSA/GRO<sub>α</sub>–97/62 and IL-8–100/65 promoter regions containing the NF-κB element are shown. The shaded boxes represent the NF-κB element and either the NF-IL6 or the IUR in the IL-8 and MGSA/GRO<sub>α</sub> promoter, respectively. Nucleotides that are not conserved between the indicated MGSA/GRO<sub>α</sub> promoter fragment and each consensus site are indicated by dots. The consensus sequences of C/EBP binding sites are IL-6 (43), IL-8 (36), albumin DE 1 (51), and serum amyloid A (SAA3) (52). The consensus RelA sequences are NF-κB p65 consensus (62), IL-8 (36), immunoglobulin κ light chain enhancer (Igκ +5) (63). The HIV terminal repeat LBP-like binding sequence is included (53, 54).
that although there were nucleotide similarities between the NF-IL-6 element (38, 39). Moreover, these results indicated bound weakly to the IL-8 enhancer region as compared to the MGSA/GRO\(\alpha\) consensus elements, the MGSA/GRO\(\alpha\) 3062 region and previously identified C/EBP consensuses. The MGSA/GRO\(\alpha\) region adjacent to the NF-\(\kappa\)B element did not bind C/EBP\(\beta\) proteins.

Identification of IUR-bound Complexes by Gel Mobility Shift Analysis—Since the majority of the nuclear complexes that recognized the MGSA/GRO\(\alpha\) 97/62 region were NF-\(\kappa\)B-related, a 42-base pair probe was prepared that contained two copies of the IUR without the NF-\(\kappa\)B element present (designated MGSA\(\alpha\) 2xIUR). When this probe was used in gel mobility shift assays with nuclear extracts from either RPE or Hs294T cells, two complexes bound (IUR-F) (Fig. 6, A and B). Cytoplasmic extracts demonstrated the presence of a slower migrating complex that appeared to be nonspecific in that all MGSA/GRO\(\alpha\) oligonucleotides tested including wild-type and mutants removed this complex in competition analysis. Unlabeled competitor DNA was used to test the specificity of the nuclear IUR-F complexes bound to the labeled MGSA\(\alpha\) 2xIUR. An oligonucleotide from the IL-8 promoter containing the C/EBP and NF-\(\kappa\)B elements did not compete (Fig. 6, lane 10).

Expression of the MGSA/GRO\(\alpha\) Gene

![Graph A](image1)

**FIG. 3.** IUR is required for MGSA/GRO\(\alpha\) promoter activity in RPE and Hs294T cells. IUR is required for MGSA/GRO\(\alpha\) promoter activity in RPE and Hs294T cells. RPE (A) or Hs294T (B) cells were co-transfected with 10\(\mu\)g of the indicated MGSA\(\alpha\)CAT construct and 2\(\mu\)g of pCMVhGH as described under "Materials and Methods." Approximately 24 h after transfection, the cells were either unstimulated (solid bars) or stimulated with 5 units/ml IL-1 for 24 h (striped bars) prior to collection. Results for RPE cell transfection are expressed as -fold activation over cells transfected with MGSA\(\alpha\)CAT alone with no treatment. -Fold induction, standard deviation of error, and number of separate transfections were MGSA\(\alpha\)CAT unstimulated (1.00) IL-1 (5.18 \pm 1.65) (n = 5), mutant IUR MGSA\(\alpha\)CAT unstimulated (0.26 \pm 0.18) IL-1 (0.46 \pm 0.26) (n = 4), mutant NF-\(\kappa\)B MGSA\(\alpha\)CAT unstimulated (0.40 \pm 0.28) IL-1 (0.41 \pm 0.18) (n = 5), and double mutant MGSA\(\alpha\)CAT unstimulated (0.04 \pm 0.02) IL-1 (0.05 \pm 0.03) (n = 3). Results for basal promoter activity in the Hs294T cells are expressed as percent CAT conversion. Percent conversions and standard error from three separate transfections were MGSA\(\alpha\)CAT wild-type (7.22 \pm 1.19), mutant IUR (0.09 \pm 0.07), and mutant NF-\(\kappa\)B (0.70 \pm 0.28).

![Graph B](image2)

**FIG. 4.** Characterization of nuclear proteins that bind to the MGSA/GRO\(\alpha\) 97/62 region. Characterization of nuclear proteins that bind to the MGSA/GRO\(\alpha\) 97/62 region. Nuclear extracts (5 \(\mu\)g) from unstimulated (NT) or IL-1 stimulated (IL-1) RPE cells were incubated 20 min at room temperature with either labeled wild-type (WT), mutant IUR (m.IUR), mutant NF-\(\kappa\)B (m.\(\kappa\)B) or double mutant (m.IUR+m.\(\kappa\)B) MGSA\(\alpha\) 97/62 oligonucleotides. Preimmune (PI) antisera or antisera to NF-\(\kappa\)B p50, NF-\(\kappa\)B p52, RelA, or C/EBP\(\beta\) were incubated with extracts prior to addition of labeled probe. The resulting protein-DNA complexes were separated on 0.5 x TBE polyacrylamide gels. The NF-\(\kappa\)B p50/RelA heterodimer and RelA complexes are indicated.

![Graph C](image3)

**FIG. 5.** Characterization of C/EBP-like elements in the MGSA/GRO\(\alpha\) and IL-8 promoter. Characterization of C/EBP-like elements in the MGSA/GRO\(\alpha\) and IL-8 promoter. Recombinant C/EBP\(\beta\) protein was incubated with 100 \(\mu\)g of \(^{32}\)P-labeled NF-IL6 oligonucleotide probe (40,000 cpm) as described under "Materials and Methods." Unlabeled competitor oligonucleotide probes NF-IL6, MGSA\(\alpha\) 97/62, and IL-8-101/-63 were preincubated 15 min prior to the addition of labeled probe. The -fold excess of each unlabeled competitor DNA is indicated. The resulting DNA-protein complexes were separated on 0.25 x TBE polyacrylamide gels. Quantitation of bound protein-DNA complexes was done using the software program IQ3.29 for the Phosphorimager (Molecular Dynamics).
cells were incubated in the presence of labeled MGSA basal and cytokine-induced MGSA/GRO complexes (Fig. 6A). Proteins to these transcription factors had no effect on the bound DNA binding elements including C/EBP and LBP sites, although the IUR sequences were similar to several consensus sequences. Purines (Fig. 6B) did not compete, while the WT and MT 2 effectively removed the bound complexes (lanes 6 and 10). Collectively, these data indicated that the IUR included the sequence TCGAT located at position -97 to -93.

Activation of MGSA/GROα Promoter by RelA—Previous work has demonstrated a cross-coupling of NF-κB and C/EBP family members (39, 59). In particular, these investigators demonstrated that regulation of the chemokine IL-8 gene expression relied on the ratio of NF-κB and C/EBP complexes in that the C/EBP protein had an inhibitory effect through the adjacent NF-κB element, while RelA enhanced transactivation through the C/EBP element (40). We were interested in determining if RelA regulated MGSA/GROα gene expression similarly through the NF-κB and adjacent regions in a manner similar to that demonstrated for the IL-8 gene (38-40). RPE cells were transiently co-transfected with a RelA expression vector, and the MGSAα350/CAT reporter constructs with point mutations in the IUR and NF-κB element. Overexpression of RelA increased activity through both the MGSAα350/CAT and the mutant IUR MGSAα350/CAT (Fig. 7). Moreover, RelA also increased transactivation through the mutant NF-κB MGSAα350/CAT. The transactivation seen with the mutant NF-κB construct was less than that observed with the mutant IUR construct. Furthermore, a second set of mutations in the NF-κB element (GAAAATTTGGC) located within the context of MGSAα350/CAT indicated that RelA still significantly increased promoter activity (data not shown). RelA expression did not transactivate through either the double mutant MGSAα350/CAT or parental pPLFCAT vectors, suggesting that the RelA transactivation observed with the mutant NF-κB element construct was through the adjacent IUR region (Fig. 7).

To address whether RelA directly bound to the MGSA/GROα IUR, RelA produced by transfected Jurkat T-cells was used in gel mobility shift analysis. RelA specifically recognized the MGSA/GROα and IL-8 NF-κB elements, although not the mutated MGSA/GROα NF-κB element. Furthermore, RelA does not bind to the MGSA/GROα IUR nor enhance the binding ability of the IUR bound complexes present in nuclear extracts (data not shown). These data suggest that RelA indirectly affects transactivation through the MGSA/GROα IUR.

IUR Contributes to Basal MGSA/GROα Expression—Gel
shift analysis indicated that several key nucleotides in the IUR were essential for recognition by the constitutive complexes in both RPE and Hs294T melanoma cells. Furthermore, these point mutations strongly affected basal and cytokine-induced MGSA/GROα/CAT activity, suggesting that the bound IUR complexes, in addition to the NF-κB complexes, contributed to the transcriptional regulation of MGSA/GROα. Minimal promoter constructs containing a single copy of the IUR plus the NF-κB element (MGSAα−97/−62TATA) or two copies of the wild type (MGSAα 2xIUR/TATA) or mutated IUR (MGSAα m.2xIUR/TATA) were generated. Transient transfections in RPE cells demonstrated that both the wild-type MGSAα−97/−62 and MGSAα 2xIUR/TATA constructs had a higher level of basal promoter activity compared with the parental TATA/CAT (Fig. 8). Furthermore, point mutations in the IUR, which resulted in loss of the constitutively bound IUR complexes effectively eliminated all basal promoter activity (Fig. 8). Together with the endogenous MGSA/GROα/CAT promoter analyses, these data indicated that the IUR-bound complexes significantly contributed to MGSA/GROα basal promoter activity.

Identification of IUR-bound Complexes by UV Cross-linking—To further characterize the complexes bound to the IUR, UV cross-linking studies were performed. Nuclear proteins bound to the labeled MGSAα 2xIUR in the gel shift reactions were either left untreated or exposed to short-wave UV irradiation for 15 min. Present in the reaction was 50-fold excess of either the wild-type or mutant MGSAα 2xIUR oligodeoxynucleotides. Half of the reaction was separated on a native 6% polyacrylamide gel (Fig. 9A). The wild-type MGSAα 2xIUR oligodeoxynucleotide (WT) effectively competed the upper bound complexes, while the mutant MGSAα 2xIUR oligodeoxynucleotide (MT) did not. UV radiation did not affect the bound complexes or the competition analysis (Fig. 9A, compare lanes 1–3 to lanes 4–6). The remaining half of the binding reaction was separated on a 9% SDS-polyacrylamide gel (Fig. 9B). Two complexes were cross-linked to the labeled probe and appeared to be specific in that the wild-type oligodeoxynucleotide significantly lessened their presence, while mutant oligodeoxynucleotide did not (Fig. 9B, lanes 13). These complexes were not observed when the binding reactions were not exposed to UV radiation (Fig. 9B, lanes 4–6). In these cross-linking studies, there were not specific complexes detected below the 40-kDa marker on the SDS-polyacrylamide gel (data not shown). The cross-linked complexes observed were approximately 68 and 50 kDa; however, the 42-base pair oligo-

Expression of the MGSA/GROα Gene

Expression of the closely related chemokine gene MGSA/GROα in several cell types is similar to the IL-8 gene. Northern analyses demonstrate that the Hs294T melanoma cells have a high constitutive level of both IL-8 and MGSA/GROα that is further induced by IL-1. Normal RPE cells have a very low constitutive level of MGSA/GROα and IL-8 expression; however, IL-1 stimulation markedly increases MGSA/GROα and IL-8 within 2 h. Previous data have demonstrated that the NF-κB element is essential for both IL-8 and MGSA/GROα promoter activity (15, 35–37). Furthermore, IL-8 gene regulation requires the C/EBP site adjacent to the NF-κB site for complete cytokine induction (36, 38, 40). Similar to IL-8, the MGSA/GROα promoter contains a region adjacent to the NF-κB element that is necessary for basal activity in both RPE and the Hs294T melanoma cells. Loss of either the IUR or the NF-κB element eliminates most of the endogenous promoter activity. Cytokine-induced MGSA/GROα/CAT promoter activity in RPE cells also requires both the NF-κB element and the IUR. However, we demonstrate here that the regions adjacent to NF-κB for the IL-8 and MGSA/GROα chemokines differ markedly in their capacity to bind transactivating factors. For IL-8, the C/EBP-like consensus sequence binds several C/EBP proteins, although with weaker affinity than established C/EBP sites. In contrast, although there is sequence similarity to a C/EBP enhancer, C/EBP proteins do not bind the IUR in the MGSA/GROα promoter. Moreover, several of the essential nucleotides in the IUR are located upstream from the C/EBP-like region.

Gel shift analyses demonstrate that it is difficult to detect a factor specific for the IUR using nuclear extraction procedures that give optimal NF-κB binding. This may in part be due to the observations that the IUR complexes require a more vigorous extraction procedure, which negatively affects NF-κB binding, and that the IUR complexes are labile after nuclear extract collection (data not shown). Alternatively, the failure to detect

FIG. 8. Basal promoter activity through MGSA/GROα/CAT. RPE cells were transfected with 10 μg of the indicated TATA/CAT construct. Transfection efficiencies were normalized by immunodetection of secreted growth hormone. At 48 h after transfection, whole cell extracts were collected as described under “Materials and Methods.” Results are relative to CAT activity for parental TATA/CAT, to which a value of 1.0 was assigned. Values with standard deviation of error from duplicates of three separate experiments were TATA/CAT (1.0), MGSAα−97/−62/TATA (6.25 ± 2.30), MGSAα 2xIUR/TATA (9.40 ± 3.50), and mutant IUR MGSAα 2xIUR/TATA (0.50 ± 0.42).

FIG. 9. UV cross-linking analysis of IUR bound complexes. Nuclear extracts (5 μg) from IL-1 stimulated RPE cells were incubated in 20 μl of total reaction volume with radiolabeled MGSAα 2xIUR oligodeoxynucleotide in the presence or absence of WT or mutant (MT) MGSAα 2xIUR for 20 min at room temperature. The binding reaction was either left at room temperature (0′) or exposed to UV radiation for 15 min (15′). A, half of the binding reaction (10 μl) was separated on a 6% 0.5 × TBE native polyacrylamide gel. The specific IUR (IUR-F) and nonspecific (NS) complexes are indicated. B, the remaining binding reaction volume was separated on a 9% SDS-polyacrylamide gel. Molecular weight standards are indicated as are two specific complexes (approximately 68 and 50 kDa) cross-linked to the labeled MGSAα 2xIUR oligodeoxynucleotide (designated I and II).

DISCUSSION

Expression of the MGSA/GROα/CAT activity for parental TATA/CAT, to which a value of 1.0 was assigned. Values with standard deviation of error from duplicates of three separate experiments were TATA/CAT (1.0), MGSAα−97/−62/TATA (6.25 ± 2.30), MGSAα 2xIUR/TATA (9.40 ± 3.50), and mutant IUR MGSAα 2xIUR/TATA (0.50 ± 0.42).

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specific IUR complexes from nuclear extracts may be due to an unstable interaction with the labeled promoter regions. Specific MGSA/GROα IUR-bound complexes are readily observed in vitro from both RPE and Hs294T nuclear extracts when two copies of the IUR are present, suggesting an increased affinity with duplicate copies. The observations that a single IUR element can compete for complex binding yet not detectably bind nuclear factors in gel shift assays suggest that the protein complexes bound to a single IUR may be unstable during electrophoresis.

The IUR complex bound to the endogenous MGSA/GROα activation in that it is able to transactivate through both the unstable interaction with the labeled promoter regions. Specifically IUR complexes from nuclear extracts may be due to an unstable interaction with the labeled promoter regions. This sequence does not have any similarities to known transcription factor sequences, suggesting that the factors that bind to the IUR are novel. UV cross-linking indicates the presence of at least two proteins of approximately 22 and 40 kDa bound to the MGSA/GROα IUR. These IUR-bound complexes are constitutively present, and IL-1 induction does not further increase the binding activity.

In addition to the NF-κB element, RelA is able to transactivate through the IUR region within the MGSA/GROα promoter. These observations are similar to those observed with IL-6 and IL-8 gene regulation in that both the adjacent C/EBP-like and NF-κB elements are essential for cytokine induction and RelA transactivation (40). However, our results differ in that RelA alone is able to transactivate the MGSA/GROα promoter and does not require an additional factor to be co-transfected.

The RelA induction through the IUR alone does not dictate that RelA cross-interacts with the IUR-bound complexes as demonstrated for the IL-8 gene. RelA antisera has no effect on the IUR-bound complexes nor does RelA enhance the binding of the IUR complexes; therefore, a direct interaction between NF-κB and IUR-bound complexes is not currently supported by our data. The IUR complexes do significantly contribute to MGSA/GROα basal promoter activity in that loss of the IUR complexes bound to the endogenous MGSA/GROα promoter, or to a minimal promoter containing only the IUR sequences, dramatically decrease the amount of activity obtained from the native promoter. RelA may act indirectly to either induce expression of the IUR binding proteins or stabilize their interaction with the basal transcription machinery.

In summary, our results indicate that, as with other genes encoding proteins involved in the inflammatory response including IL-6 (40), IL-8 (36, 38, 39), serum amyloid A genes (52, 60), and angiotensinogen (61), MGSA/GROα transcriptional regulation requires multiple factors recognizing the NF-κB and adjacent DNA binding elements. However, the IUR adjacent to the NF-κB element in the MGSA/GROα promoter appears to be unique. In addition, RelA has a dual role in MGSA/GROα activation in that it is able to transactivate through both the NF-κB element and the adjacent IUR. Since NF-κB is ubiquitous and a multitude of genes contain NF-κB elements, the regulation by RelA through a separate enhancer region may allow a tighter and more specific level of gene regulation. Future studies are needed to determine the mechanism by which this region interacts with the adjacent NF-κB element to regulate MGSA/GROα transcription in normal and transformed cells.

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