BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 56–62 (1998)
ARTICLE NO. RC989594

Characterization of the Mouse Interleukin-5 Promoter in a Mouse TH2 T Cell Clone

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Received September 17, 1998

The proximal mouse IL-5 promoter was examined using a mouse TH2 clone stimulated through the T cell receptor using anti-CD3 monoclonal antibody. DNase I protection defined four protein binding regions [IL-5RE-A, -69/-45; -B, (-90/-76); -C, (-154/-130); and -D (-176/-157)]. Stimulation-dependent binding, which was seen in the IL-5RE-B, -D regions and the 5′ end of tIL-5RE-A, did not require new protein synthesis inhibitor during cell activation. EMSA using probes targeted to the IL-5RE-B, -C, -D regions demonstrated the multimeric nature of the bound proteins. By transfection analysis using a series of truncated IL-5 promoter-luciferase constructs, IL-5RE-C and -D contributed little to constitutive or inducible activity. The CLE0 site in the IL-5RE-A region contributed to full transcriptional activity but was not sufficient to mediate full activity. Full stimulation-dependent activity required the IL-5RE-B region and/or the GATA site (-70/-60).

Interleukin-5 (IL-5), a cytokine produced primarily by T helper 2 (TH2) cells, selectively induces the growth and differentiation of eosinophils from bone marrow precursors (1). Eosinophils predominate in the inflammatory cell infiltrate observed in animal models of pulmonary inflammation (2,3),which mimic the pathophysiology of asthma. The therapeutic use of anti-IL-5 antibodies in these animal models has established the importance of IL-5 in the cascade of events which results in eosinophil inflammation in the lungs of allergic animals (4-6).

In this study we describe further analysis of the elements of the mouse IL-5 promoter which control IL-5 transcription. This analysis employed nontransformed mouse TH2 cells stimulated through the T cell receptor (TCR) as a source of both nuclear extracts and as transfection targets. This system differs significantly from others where pharmacologically stimulated transformed thymoma cells (EL-4) were used (7-10). In addition, much prior work focused on the Conserved Lymphokine Element 0 (CLE0) in the EL-4 system (7-10) as well as in nontransformed T cells (11,12). To further our knowledge of IL-5 transcription we sought to 1) define novel protein binding regions in the proximal IL-5 promoter by DNase I protection and electrophoretic mobility shift assays (EMSA); and 2) specifically correlate those regions with functional activity by transfection analysis. Because a distinguishing feature of inducible IL-5 mRNA expression compared to other cytokines is its critical dependence on protein synthesis (13-17), we sought to determine if specific protein binding regions defined above could be correlated with a dependency on new protein synthesis.

MATERIALS AND METHODS

T cells. The murine $CD3^+$, $CD4^+$, $CD8^-$ Th2 T cell clone D10.G4.1 (18)(ATCC, Rockville, MD) (conalbumin specific, H-2 $I\text{-}A^k$ restricted) was used as a source of nuclear extracts and for transfections. D10.G4.1 cells were maintained in culture as described previously (15). The cells were used for nuclear protein preparations and electroporation experiments on day 8 of this 14 day antigen-stimulated growth cycle.

Preparation of nuclear extracts. Nuclear extracts were prepared from unstimulated D10.G4.1 cells, stimulated cells, and cells that were stimulated in the presence of cycloheximide (CHX). The cells were stimulated through the TCR with anti-CD3 monoclonal antibody (α-CD3 mAb) as previously described (19). Unstimulated cells did not produce detectable IL-5, while α-CD3 mAb stimulation induced IL-5 gene expression and protein secretion. Where indicated, the cells were pretreated for 10 min with 10 μg/ml CHX (Sigma) prior to antibody stimulation. The nuclear proteins were isolated by a modification (19) of the procedure described (20). The protein content of the nuclear extracts was determined by the method of Bradford (21).

Electrophoretic mobility shift assays (EMSA). Complementary oligonucleotide pairs corresponding to mouse IL-5 5′ flank sequences with 5′-GGG overhanging ends were synthesized (Life Technologies, Inc., Gaithersburg, MD; Oligos,Etc, Inc., Wilsonville, OR)) annealed, and radiolabeled with [α-^{32}P]-dCTP (19). The sequences of the positive strand for each oligonucleotide pair (without the 5′-GGG over-

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0006-291X/98 $25.00

hanging ends) are shown in Fig. 2A. The preparation of labeled, annealed, purified oligonucleotides, EMSA binding reactions and competition assays for determining protein-DNA binding specificity were performed as described previously (19).

Mouse IL-5 promoter constructs. The cloning by PCR and construction of the 584 bp mouse IL-5 promoter region in the pGL2basic luciferase reporter plasmid, designated MuIL5(-546)luc, has been described (15). Murine IL-5 promoter reporter constructs truncated at positions -177, -58, and -40 relative to the start of transcription at position +1 have also been described (15). Additional deletion constructs truncated at positions -152 and -121 were generated by PCR amplification using primer IL5-3′ (15) and one of the following 5′ primers: IL5-152: (GGGAGCTCAAAAAAAAAAATGCATTTGT); IL5-121: (GGGAGCTCAGGGCACTGGAAACCCTGAG). The PCR products were subcloned into the *Sac*I/*Bgl*II sites in the pGL2basic vector to create the IL-5 promoter luciferase gene reporter constructs. All constructions were verified by DNA sequencing. The cloning of the IL5RE-B deletion construct was accomplished using two complementary 76 base oligonucleotides (Oligos, Etc.) containing the sequence between positions -186 and -125 of the mouse IL-5 promoter, a *Kpn*I restriction sequence at one end, and a *Sac*I restriction sequence at the other end. This IL-5 promoter sequence includes the IL-5RE-D and -C regions. The complementary oligonucleotides were annealed, digested with *Kpn*I and *Sac*I, and cloned in the *Kpn*I/*Sac*I site of the Mu-58 construct described previously (15). The deletion construct that resulted was sequenced to confirm that it contained the IL-5 promoter -186/-125 region upstream of promoter position -58. All constructs were depicted graphically in Fig. 2A.

Transfection assays. D10.G4.1 T cell clones were transfected with the indicated reporter gene constructs by electroporation and the relative light units (RLU) of luciferase activity in each sample were assayed (19). The MuIL5(-546)luc construct was used as the positive control and the promoterless pGL2basic vector was used as a negative control in each of the transfection experiments. The background level of luciferase activity using only lysate buffer was subtracted from the luciferase values of each sample. Typical luciferase measurements from stimulated cells transfected with the MuIL5-(-546)luc construct were 20,000-50,000 RLU. The luciferase activity of each transfected construct with or without α-CD3 mAb stimulation was compared to α-CD3 mAb-stimulated cells transfected with the MuIL5(-546)luc construct (set at 100%) to obtain the RLU activity for each construct in each independent transfection experiment. The transfection results presented represent the mean ±/− SEM of 4-6 independent experiments with each construct.

DNase I protection assays. DNase I footprinting was performed as described by Galas and Schmitz (22) with modifications (19). The double-stranded DNA templates were created by sequential digestion of the MuIL5(-261)luc construct with either *Bgl II* followed by *Sac I* to create the noncoding strand template or *Xma* I followed by *Eco 47*III to create the coding strand template. Cycle sequencing reactions for the coding and noncoding strands were used to orient the DNase I footprint regions within the template sequence. All cycle sequencing reactions were carried out with the dsDNA Cycle Sequencing System (Life Technologies) using twenty bp oligonucleotides complementary to the 5′ end of each template strand as primers. The template used was the same as that used for the DNase I footprinting reactions.

RESULTS

The Proximal Mouse IL-5 Promoter Contains Four DNA–Protein Binding Regions

DNase I protection assays with the noncoding strand template of the murine proximal IL-5 promoter and nuclear extracts from each of three cell treatment groups are shown in Fig. 1. The cell treatments were no stimulation, stimulation with α-CD3 mAb, or stimulation with α-CD3 mAb in the presence of the protein synthesis inhibitor, CHX. Four distinct footprints, designated IL-5 Response Element-A (IL5RE-), B, C, and D, in addition to the TATA sequence, were protected from DNase I digestion with each of the nuclear extract preparations. Delineation of the nucleotides encompassed within each protected region is also depicted in Fig. 2A. The protected site closest to the TATA sequence in the IL-5 promoter was designated IL-5RE-A and was located between positions -69 and -45. The

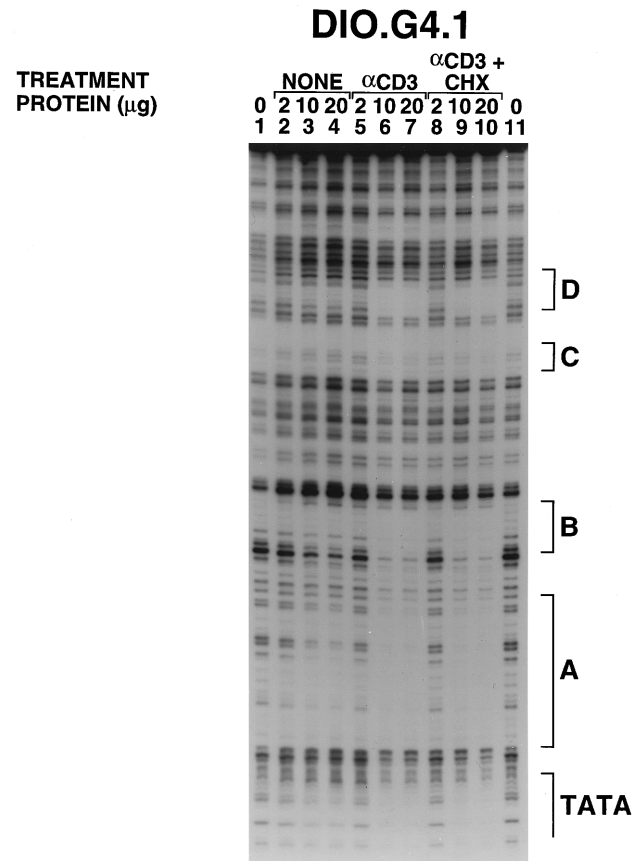

FIG. 1. DNase I protection assays define four footprinted regions in the mouse IL-5 promoter. Nuclear extracts were prepared from D10.G4.1 cells that were unstimulated, stimulated by α-CD3 mAb, or stimulated by α-CD3 mAb in the presence of CHX as detailed under "Materials and Methods." The IL-5 promoter template spanning from −261 to +39 relative to the transcription start was prepared and labeled at the +39 terminus as also described under "Materials and Methods." The regions that are protected from DNase I digestion in the presence of nuclear extracts are indicated by brackets. lanes 1 and 11 are replicate digestions of the noncoding strand in the absence of nuclear protein; lanes 2–4, lane 5–7, and lanes 8–10 represent the noncoding strand digested in the presence of the indicated amount of nuclear protein from unstimulated D10.G4.1 cells, α-CD3 mAb stimulated cells, or from cells stimulated by α-CD3 mAb in the presence of CHX, respectively. The ddG, ddA, ddT, and ddC cycle sequencing reactions used to align the protected regions with the template sequence are not shown. DNase I protection assays performed with the coding strand template are not shown.

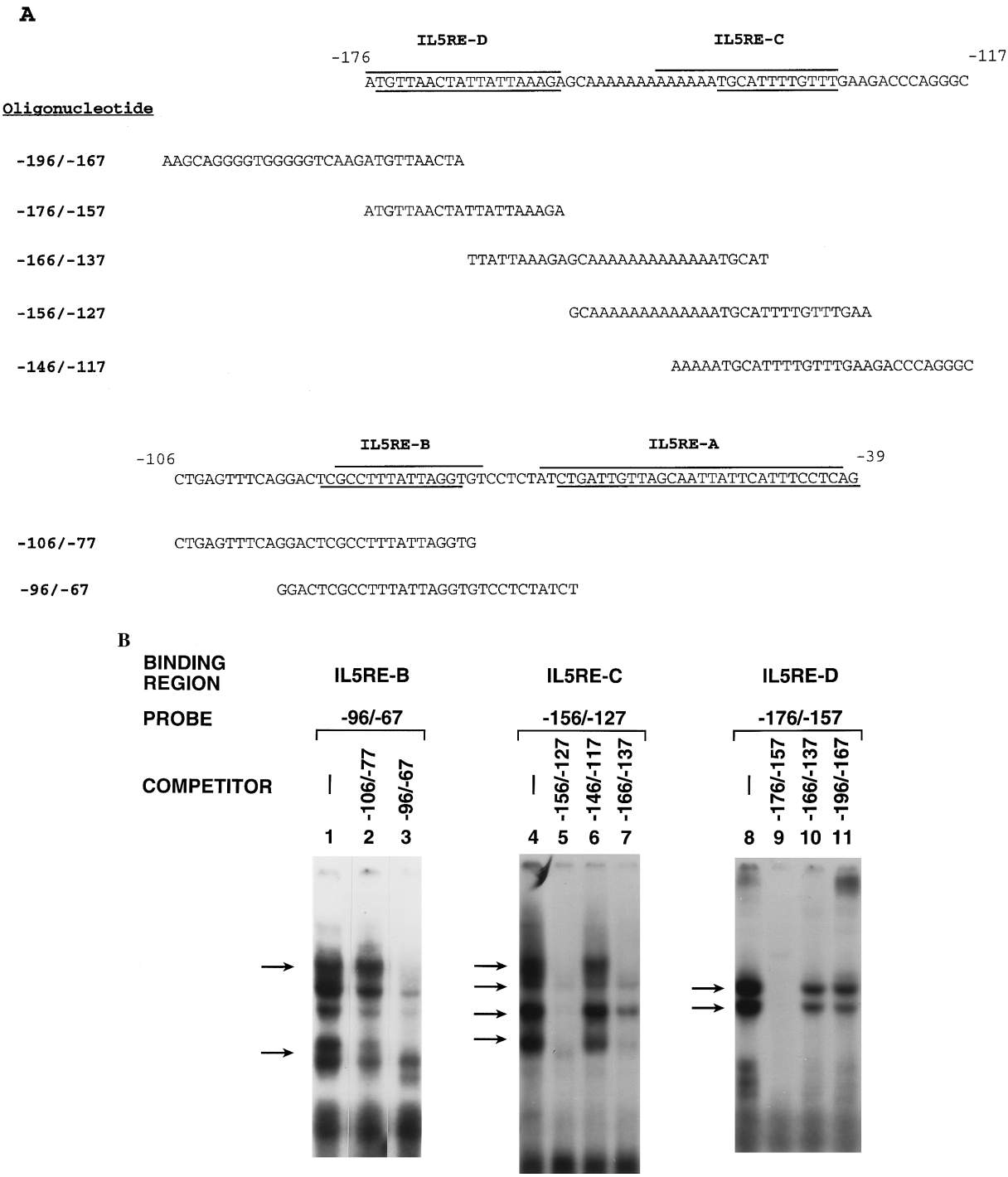

FIG. 2. Electrophoretic mobility shift assay demonstrating protein binding to IL-5RE-B, -C, -D regions of the mouse IL-5 proximal promoter. A. Overlapping oligonucleotide probes and competitors used in EMSAs are shown. Positive strand sequences of the duplex oligonucleotides used as either probes or unlabelled competitors are indicated with their positions relative to the transcription start site at +1 in the mouse IL-5 promoter. The relative protein binding regions IL5RE-D, IL5RE-C, IL5RE-B, and IL5RE-A, as defined by DNAse I protection, are also shown. Upper line denotes the positive strand protected nucleotides; lower line denotes the negative strand protected nucleotides. The location of each footprint is indicated by the numbers and is relative to the transcription start site at +1. The actual bases on each strand that are protected from digestion are contained within the brackets. B. Nuclear protein extracts were prepared from D10.G4.1 cells stimulated by plate-bound α-CD3 mAbs as described under "Materials and Methods." Each binding reaction contained 5 μg of nuclear protein and the indicated labeled probe encompassing one of the IL-5RE regions as indicated. Excess unlabeled specific or adjacent competitor oligonucleotides as noted were preincubated with the nuclear extracts prior to the addition of probe. The protein–DNA complexes were resolved by PAGE on a nondenaturing 6% acrylamide gel. Specific retarded labeled complexes are indicated by arrows.

most prominent protection of this -69 to -45 bp region was at the 5′ end and was provided by nuclear extracts from α-CD3 mAb stimulated cells (Fig. 1, lane 7). Protection from digestion was less evident with unstimulated cell extracts (Fig. 1, lane 4). This region overlaps a GATA site (23). In contrast, the 3′ end of the IL-5RE-A region, which overlaps the CLE0 (15,24,25)) sequence (-59/-36), was equally protected from digestion by proteins from either unstimulated or stimulated D10.G4.1 cells. This region also includes sequences with some homology to a number of regulatory sites, namely AP-1 (25), NF-IL6 (26), and CATTT (25) previously identified in the promoter regions of several cytokine genes.

The protected site designated IL-5RE-B in Fig. 1 was located between positions -90 and -76. The protection of this region was strongly dependent on inducible proteins, since in the absence of cell stimulation, nuclear proteins did not footprint this region (lanes 2-4). A protected region was also observed between positions -154 and -130 and was designated IL-5RE-C. The footprint in the IL-5RE-C region was only weakly evident even with increasing amounts of nuclear protein (Fig. 1, lanes 2-11) and protection of this region was not stimulation dependent. In the absence of nuclear protein, however, the template in this region was inefficiently digested by DNase I (lanes 0 and 11), suggesting that this area of the promoter is poorly accessible for protein interactions; exact boundaries of IL-5RE-C were difficult to determine. Nonetheless, this site was consistently footprinted to some extent in multiple independent experiments with both template strands and was therefore designated as a protein binding site. Both IL-5RE-B and -C, did not contain significant sequence homology to known cytokine regulatory sites.

The most distal footprint evident in the promoter sequence was designated IL-5RE-D and was located between positions -176 and -157 (Fig. 1). Similar to the IL-5RE-B site, the protection of this region was dependent on the activation of the cells and was concentration dependent (lanes 5-7). In the absence of stimulation, this region was not protected from digestion (lanes 2-4). The pattern of protection seen with the stimulated cell extracts in IL-5RE-A, -B, -C, and -D was unaltered when nuclear protein from cells stimulated in presence of protein synthesis inhibitor, CHX, were included in the footprinting reaction (lanes 8-10 vs 5-7).

EMSAs were performed with a series of ^{32}P-labeled oligonucleotides, targeted to the newly described IL-5RE-B, -C, -D regions (Fig. 2) and nuclear extracts from α-CD3 mAb stimulated D10.G4.1 cells. The oligonucleotides used as probes and competitors and their positions relative to the transcription start site at +1 and the IL-5 RE-A, -B, -C, and -D footprints are shown in Fig. 2A. Because several prior studies have described the protein complexes that bind to the CLEO site contained in the IL-5RE-A region (7,9,12,15), this region was not examined.

Using the oligonucleotide probe -96/-67, which spans the IL-5RE-B region, multiple distinct protein complexes of different sizes were retarded in their mobility (Fig. 2B, lane 1). None of these complexes were efficiently competed with excess unlabeled oligonucleotide, containing the partially overlapping sequence between positions -106 and -77, (lane 2). However, at least two of these retarded complexes (marked by arrows) were specifically competed in the presence of excess unlabeled -96/-67 oligonucleotide (lane 3).

To examine the IL-5RE-C region, the -156/-127 oligonucleotide was used as labelled probe (Fig. 2B, lanes 4-7). At least four protein complexes were retarded with this probe spanning the IL-5RE-C region (Fig. 2, lane 4). All of these complexes were shown to be specific for this binding region by the absence of complex formation with unlabeled -156/-127 oligonucleotide as competitor. No significant alteration of complex formation was observed with the overlapping -146/-117 oligonucleotide as competitor (lane 6). Partial competition of these complexes was observed with the overlapping -166/-137 oligonucleotide, indicating that the 5′ end of the region protected from DNase I digestion (Fig. 1) is required to disrupt some but not all of the IL-5RE-C bound complexes.

Using the oligonucleotide probe -176/-157, including nucleotides which define IL-5RE-D (Fig. 1), at least two protein complexes (Fig. 2B, lane 8) were retarded. These complexes were specifically competed with unlabeled -176/-157 oligonucleotide (lane 9), but they were not competed in the presence of the overlapping oligonucleotides -166/-137 (lane 10) or -196/-167 (lane 11). Thus, EMSA analysis using stimulated T cell extracts and oligonucleotide probes targeted to the IL-5RE-B, -C, -D regions confirmed protein binding observed in the DNase I protection experiments and demonstrated the multimeric nature of the bound complexes.

Functional Analysis of the Mouse IL-5 Promoter

To examine the functional role that protein binding sites in the 5′ region of the IL-5 promoter, defined above, might play in controlling inducible transcription of the gene, the activity of a series of mouse IL-5 promoter-luciferase reporter gene constructs (Fig. 3A) was assessed in a transient transfection assay in D10.G4.1 T cells (Fig. 3B). The transfection assay has been demonstrated previously (12,15,27,28) to support the constitutive and inducible expression of IL-5 reporter gene constructs.

Significant luciferase activity was detected in cells transfected with the full length m-546 construct and stimulated with α-CD3 mAb, and this level of activity was 2.3 fold higher than the activity with the same

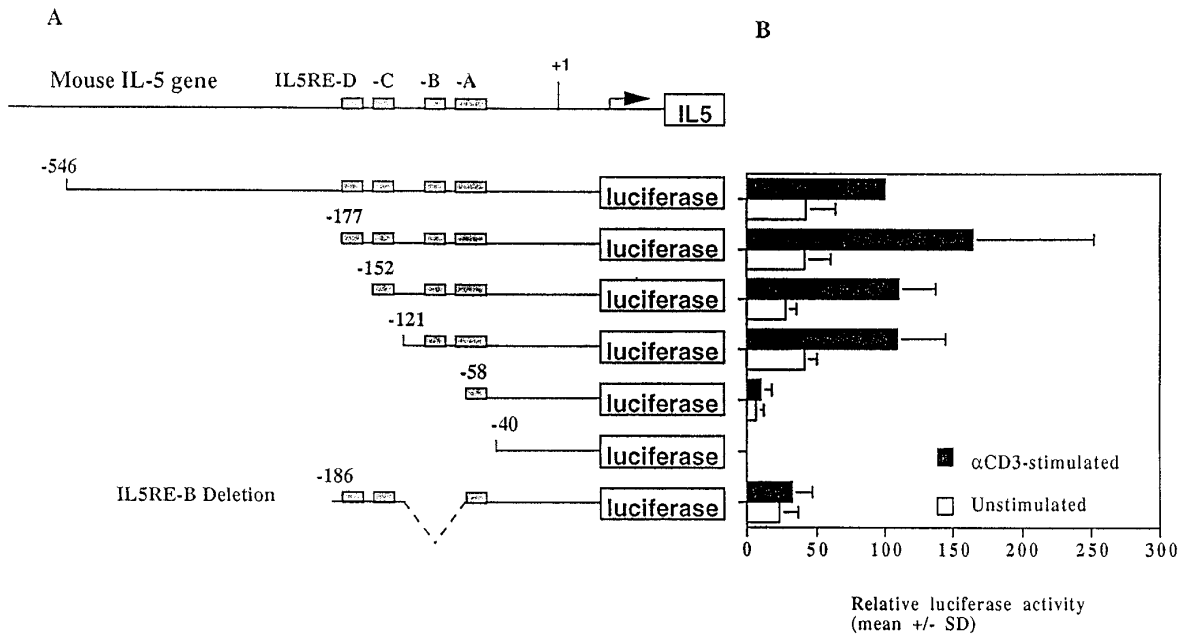

FIG. 3. Relative contribution of IL5RE-A, -B, and -D to transcriptional activity. A. A series of murine IL-5 promoter luciferase constructs was used in transient transfection experiments. The 5′ end of each construct is designated by position relative to the transcription start site at +1; the IL-5RE-B deletion construct contains the -186/-125 region upstream of promoter position -58. Shaded boxes indicate the IL-5RE protein binding sites defined by DNAse I protection (Figure 1). B. D10.G4.1 cells were transiently transfected by electroporation and cultured with or without plate-bound α-CD3 mAb stimulation; luciferase reporter gene activity was measured in the cell lysates as described in the "Materials and Methods". Each construct was tested in unstimulated and stimulated D10.G4.1 cells in parallel. The results are presented as luciferase activity (mean +/− SEM of 4-6 independent experiments) relative to the full-length IL-5 promoter construct (m-546) which was designated 100 % activity.

construct in unstimulated transfected cells. Removal of the nucleotides between positions -546 and -177 resulted in a variable increase of approximately 50% in the luciferase activity in stimulated cells transfected with the m-177 construct. This latter construct contained all four of the protein binding regions defined by DNase I protection and EMSA. Transfection with the m-152 construct, lacking the IL5RE-D region, or the smaller m-121 construct, in which the IL-5RE-C region was also deleted, resulted in luciferase levels in both unstimulated and stimulated transfected cells, that were similar to those obtained with the m-546 construct. These results indicate a minimal role for the IL-5RE-C and-D regions in either constitutive or inducible activity mediated by this promoter.

The m-58 construct was designed to assess the contribution of two potential transcription factor binding elements contained within IL-5RE-A. These elements are a region between -70 and -60 containing two overlapping consensus GATA sites (A/TGATAA/G) (7,9) and the CLE0 site between -59 and -36 of the mouse IL-5 promoter. The m-58 construct, lacked the IL-5RE-B, C and D region, and the GATA site but contained the CLEO site. In the transfections with the m-58 construct, a significant decrease in activity was noted in transfected cells stimulated with α-CD3 mAb. A loss of activity was also seen in m-58 construct transfected, unstimulated cells; a 1.5-fold induction with stimulation was observed with this construct. This construct, which contains the CLEO site, contributed to a low level of both constitutive and inducible IL-5 promoter activity, as evidenced by the absence of all activity of the m-40 construct, which lacks the CLEO site but contains the TATA site.

The IL-5RE-B Deletion construct (Fig. 3A) was designed to specifically evaluate the activity of a construct in which the IL-5RE-B region and GATA sites (-69/-58) were removed but the 5′ protein binding elements, IL-5RE-C and -D were maintained. This construct was similar to the m-58 construct, in that it retained the CLEO site but lacked the GATA site. The levels of activity (Fig. 3B) with the IL-5RE-B Deletion construct in the transfected, stimulated D10.G4.1 cells were approximately 2-fold higher than those with the m-58 construct but were lower than those with the constructs that included all four protein binding regions (m-177 and m-546). A similar pattern was obtained in unstimulated, transfected cells. Thus, the IL-5RE-B region and/or the GATA site contributed to maximum promoter activity, and the inclusion of the IL5RE-C and IL5RE-D elements could not compensate for the loss of IL-5RE-B or GATA sites in controlling the activity of the promoter. In addition, the activity levels of the IL-5RE-B Deletion construct in the trans-

fected cells was similar with or without α-CD3 mAb stimulation of the transfected cells (1.4-fold induction).

DISCUSSION

The production of IL-5 is restricted to appropriately stimulated TH2 but not TH1 T cells, is temporally regulated in those cells, and also uniquely modulated by various pharmacological agents (29-33). Its production is largely but not exclusively regulated at the level of transcription (16,34). Several studies with the mouse IL-5 promoter have used transformed EL-4 thymoma cells stimulated with cAMP and PMA as a source of nuclear protein extracts and as recipients for transfections (7-10). We have previously described several cis-acting regions in the 5′-flank of the mouse IL-5 gene that are involved in the specific and inducible regulation of IL-5 transcription in an untransformed mouse TH2 T cell clone, D10.G4.1, stimulated through the TCR (19). Here, we have continued the use of this experimental system to identify protein binding sites in the IL-5 promoter that are critical for inducible promoter activity.

Two DNA-protein binding regions which mediate significant transcriptional activation are IL-5RE-A (-69/-45) and IL-5RE-B (-96/-76). Both the IL-5RE-B and the 5′ end of IL-5RE-A are defined by strong protection from DNase I digestion and which is cell-stimulation dependent. Interestingly, known transcription factor binding sites are contained within the IL-5RE-A: a GATA site (-70/-60) and the adjacent CLE0 site (-59/-36) (15,24,25). The functional contribution of the latter site is clear from the transfection experiments in which the m-58 construct, which lacks the IL-5RE-B, -C, and -D regions and the GATA element of IL-5RE-A but retains the CLE0 site, mediates transcriptional activity that is greater than the m-40 construct lacking the CLEO site as well as the upstream IL-5RE-B, -C, -D sites. In addition, CLE0, in the context of IL-5RE-C and -D (IL-5RE-B deletion construct) mediates activity 2-fold greater than that of CLE0 alone (m-58). However, the level of activity generated by the m-58 construct is 4-fold less than that observed with the full length construct or a smaller construct that contains the IL-5RE-A, -B, -C, and -D regions (m-177). The loss of activity was noted in both unstimulated and stimulated transfected cells. Thus, CLE0 contributes to full transcriptional activity but is not sufficient alone to mediate more than only weak activity. However, the contribution of CLE0 may be stronger than that of the protein-binding regions of IL-5RE- C and -D.

The importance of the GATA site and/or additional protein binding sites within the IL-5RE-B region is suggested by the degree of transcriptional activity attained with the IL-5RE-B deletion construct lacking the GATA site. The activity of this construct was approximately 4-fold less than that observed with the full length, m-177, m-152, or m-121 constructs. The low transcriptional activity observed with the m-58 construct, which lacks the GATA site and all sites upstream of -58 bp, also supports this. Interestingly, a minimal difference in transcriptional activity between unstimulated and stimulated transfected cells was observed for both the m-58 and the IL-5RE-B deletion constructs in contrast to all other active constructs. This strongly indicates that the GATA site and/or additional sites in the IL-5RE-B region contribute significantly to full stimulation-dependent activity mediated by the TCR.

Several recent reports have described the importance of this GATA site. In supershift EMSA experiments, binding activity of GATA-3, but not GATA-4, to the -70/-60 region of the mouse IL-5 promoter was observed in nuclear extracts from transformed EL-4 thymoma cells stimulated with cAMP and PMA (9) and from PMA and ionomycin stimulated D10.G4.1 TH2 cells (11). In addition, GATA-3 mRNA is selectively expressed in mouse TH2 but not TH1 cells (11,35). Lastly, expression of GATA-3 directly activates the IL-5 promoter in PMA and ionomycin stimulated EL-4 cells cotransfected with an IL-5 promoter/reporter construct and an expression plasmid containing murine GATA-3 cDNA (11). Additional support for the importance of this GATA binding region is provided here. Strong protection from DNase I digestion of the 5′ end of IL-5RE-A, including the GATA site, was observed. The functional importance of this region was confirmed by transfection analysis of mIL-5 promoter constructs containing or lacking this site. Importantly, both lines of evidence were obtained in a physiologically relevant nontransformed T cell stimulated, not pharmacologically, but rather through the TCR.

Two more distal promoter-protein binding sites, IL-5RE-C (-154/-130) and -D (-176/-156) were also identified. The protection from DNase I digestion of the latter but not the former region was enhanced in response to T cell stimulation. However, deletion of either of these regions did not appreciably alter the degree of transcriptional activity or the enhancement of transcription in response to stimulation, compared to either the full length construct (m-546) or the shorter construct (m-177) which contained all four protein binding regions defined by the footprint assays. Thus, the functional role of IL-5RE-C and -D is unclear.

An additional observation emerges from this study. IL-5 gene transcription has previously been shown to be highly dependent on de novo protein synthesis (13-17). Our experimental design incorporated the use of nuclear extracts from CHX-treated, α-CD3 stimulated cell extracts to delineate regions in the promoter whose protein-binding pattern was altered by the blockade of new protein synthesis. No clear differences were observed in the DNase I protection pattern or complex

formation in EMSAs between CHX-treated or untreated stimulated cell extracts. The possibility remains that newly synthesized proteins are involved in post-translational modification of DNA-binding proteins that is required for IL-5 transcription but this modification is not revealed by the techniques used here.

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