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Roles of Ets proteins, NF-κB and nocodazole in regulating induction of transcription of mouse germline Ig α RNA by transforming growth factor-β1

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Abstract

Antibody class switch recombination (CSR) occurs after antigen activation of B cells. CSR is directed to specific heavy chain isotypes by cytokines and B cell activators that induce transcription from the unrearranged, or germline (GL), C_H region genes. Transforming growth factor (TGF)-β1 is essential for switch recombination to IgA due to its ability to induce transcription from GL Ig α genes. It has been shown that the promoters which regulate transcription of mouse and human GL α RNAs contain a TGF-β1-responsive element that binds Smad and core binding factor (CBFα)/AML/PEBPα/Runx. They also contain other elements which bind the transcription factors CREB, BSAP and Ets family proteins. In this manuscript we demonstrate that two tandem Ets sites in the mouse GL α promoter bind the transcription factors Elf-1 and PU.1, and that the 3′ site is essential for expression of a luciferase reporter gene driven by the GL α promoter. Binding of Elf-1 to the GL α promoter is inducible by lipopolysaccharide in nuclear extracts from splenic B cells. An NF-κB site is identified, although it does not contribute to expression of the promoter in reporter gene assays. Since CSR to IgA is greatly reduced in NF-κB/p50-deficient mice, these data support the hypothesis that NF-κB has roles in switching in addition to regulation of GL transcription. Finally, we demonstrate that nocodazole, which disrupts microtubules that sequester Smad proteins in the cytoplasm, stimulates transcription from the GL α promoter.

Introduction

Upon antigenic or mitogenic stimulation, B lymphocytes proliferate and differentiate. As they proliferate, some of the progeny switch from expression of IgM to expression of other Ig classes. Ig heavy chain class switching allows the recombined variable (VDJ) region gene segment to be expressed with a new downstream constant region (C_H) gene, resulting in a change in the effector function of the antibody. Class switching is effected by a deletional recombination which occurs between switch region sequences located upstream of each C_H gene. Class switch recombination (CSR) is directed to a particular C_H gene by cytokines which induce transcription from unrearranged, or germline (GL), C_H genes before switch recombination to the same C_H gene (1). There is a good correlation between the specificity of GL transcription and the specificity of the isotype switching events. Furthermore, targeted deletion of DNA segments containing the promoter and first exon (I exon) of a GL transcript abrogates switching to that allele (2–5). Therefore, although the molecular mechanism of CSR is unknown, the data indicate that regulation of transcription of unrearranged C_H genes is important for regulation of class switch recombination.

In order to understand how switching to IgA is regulated, we are studying the regulation of transcription of GL α RNA. Transforming growth factor (TGF)-β1 induces GL α transcripts in mouse splenic B cells activated by polyclonal activators, e.g. lipopolysaccharide (LPS), and, subsequently, cells in this

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population will undergo switch recombination to IgA (6–9). Similarly, in the I.29μ B lymphoma cell line, TGF-β1 increases GL α transcripts and in the presence of LPS induces switching to IgA (10). The induction of GL α transcripts by TGF-β1 in this cell line was shown to be due to regulation at the transcriptional level (10). Importantly, TGF-β1-deficient mice produce very little IgA (11).

TGF-β1 is a pleiotropic cytokine, having suppressive effects on growth, and both inhibitory and stimulatory effects on the differentiation of a variety of cell types (12,13). Receptors for the TGF-β superfamily have serine/threonine kinase domains, and treatment with TGF-β1 results in post-translational modification and activation of the transcription factors Smad 2 and Smad 3 (13–15). TGF-β1 also induces synthesis of other transcription factors, i.e. AP-1 (16,17) and core binding factor (CBFα) 3 (18).

To attempt to understand the regulation of transcription of GL α RNA, DNA segments containing the 5′ flank and first initiation site of the transcripts have been analyzed by reporter gene assays. A deletional analysis indicated that the DNA segment between −130 and +46, relative to the first initiation site for mouse GL α transcripts, is sufficient for expression and TGF-β1 inducibility of a reporter gene in two different B cell lines (19). Within this region, a novel element (TβRE), located at −128/−104 relative to the first RNA initiation site in the GL α promoter, was found to be required for TGF-β1 inducibility of a reporter gene and when multimerized was sufficient to transfer the inducibility to a minimal c-*fos* promoter (19). This element, which is present in both the mouse and human promoters, consists of a direct repeat unit containing binding sites for two different transcription factor families: (i) Smad and (ii) CBFα proteins (also known as Runx, AML or PEBPα) (18,20–25). Additional CBFα binding sites are also present in the mouse and human GL α promoters (18,23). When activated by TGF-β1 treatment, Smads 3 and 4 synergize with CBFα to induce the GL α promoter (22,24,25). Synthesis of one of the CBFα proteins, CBFα3, is inducible by TGF-β1 in splenic B cells and in the B cell line I.29μ (18).

A binding site for an ATF/CREB transcription factor is also important for expression and TGF-β1 inducibility of both the mouse and human GL α promoters in reporter gene assays (19,26). Recently, CREB was shown to bind this site and to synergize with Smads 3 and 4, and CBFα to confer TGF-β1 inducibility to the mouse promoter when over-expressed (25). Surprisingly, the B cell-specific protein BSAP was found to inhibit transcription when bound to a site in the GL α promoter (27), whereas it activates transcription from the GL ε promoter (28,29).

An Ets site has been shown to be essential for expression of the human GL α promoter (26), although which protein(s) functions through this site was not determined. The mouse promoter also has an Ets consensus element at a comparable position, although its function has not been previously examined. Ets sites have a core GGA sequence, with most having a GGAA core, and additional nucleotides surrounding the core affect which particular Ets protein binds at a specific site (30). However, since most cells have several different Ets proteins it is often difficult to determine which protein(s) actually binds and regulates transcription of particular genes. Furthermore, Ets proteins often require protein partners in order to bind DNA and to activate transcription (31).

In this manuscript we compare the effects of nucleotide substitutions in all the transcription factor binding sites known to be, or potentially, important for activating the mouse GL α promoter. We then focus our efforts toward identifying the Ets proteins that bind the GL α promoter in B cells and their role in transcription. We demonstrate that the Ets site is important for TGF-β1 inducibility in addition to overall activity of the promoter in I.29μ B cells. We show that although this site binds Fli-1 when present on a small DNA segment, it binds Elf-1 and PU.1 in the context of the full-length promoter, and that Elf-1 binding is inducible in splenic B cell extracts by LPS.

It has been reported that mice with targeted deletions of various NF-κB genes have defects in antibody class switching. For some isotypes, e.g. IgE, the reduction in isotype production correlates with a defect in ability to synthesize GL transcripts (32,33). For IgA, it was found that NF-κB/p50-deficient mice produced 4-fold less IgA upon immunization (32) and that splenic B cells induced in culture switched ~10-fold less well to IgA than wild-type B cells (33). However, Snapper *et al*. (33) found that there was no decrease in the levels of GL α transcripts induced in the p50-deficient mice. In this manuscript we examine the effect of mutation of a NF-κB binding site on the activity of the GL α promoter.

Methods

Cell lines

Two mouse B lymphoma cell lines were used in this study: I.29 μ (22D subclone), an sIgM$^+$ B cell line which can be induced to undergo class switch recombination to IgA (34,35), and A20.3, a sIgG2a$^+$ B cell line which has not been demonstrated to undergo class switching (36).

Cell culture

I.29μ cells were cultured as described (10). A20.3 cells were cultured at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 mg/ml kanamycin sulfate (the last six reagents came from Gibco, Grand Island, NY). In transfection experiments with I.29μ cells shown in Fig. 1, human or porcine TGF-β1 (R & D Systems, Minneapolis, MN) was added twice (right after transfection and again at 7 h after transfection) at a concentration of 1 ng/ml each time. In experiments with A20.3 cells and in all other experiments with I.29μ, TGF-β1 was added right after transfection at a final concentration of 2 ng/ml. The amount of TGF-β1 used was determined to be in the optimal range (data not shown).

Splenic B cells were purified and cultured as described (37), except cells were cultured for 14 h with TGF-β1 (2 ng/ml) and LPS (50 μg/ml).

Oligonucleotides

The sequences of the top strand of the double-stranded oligonucleotides used in electrophoretic mobility shift assays (EMSA) are given below.

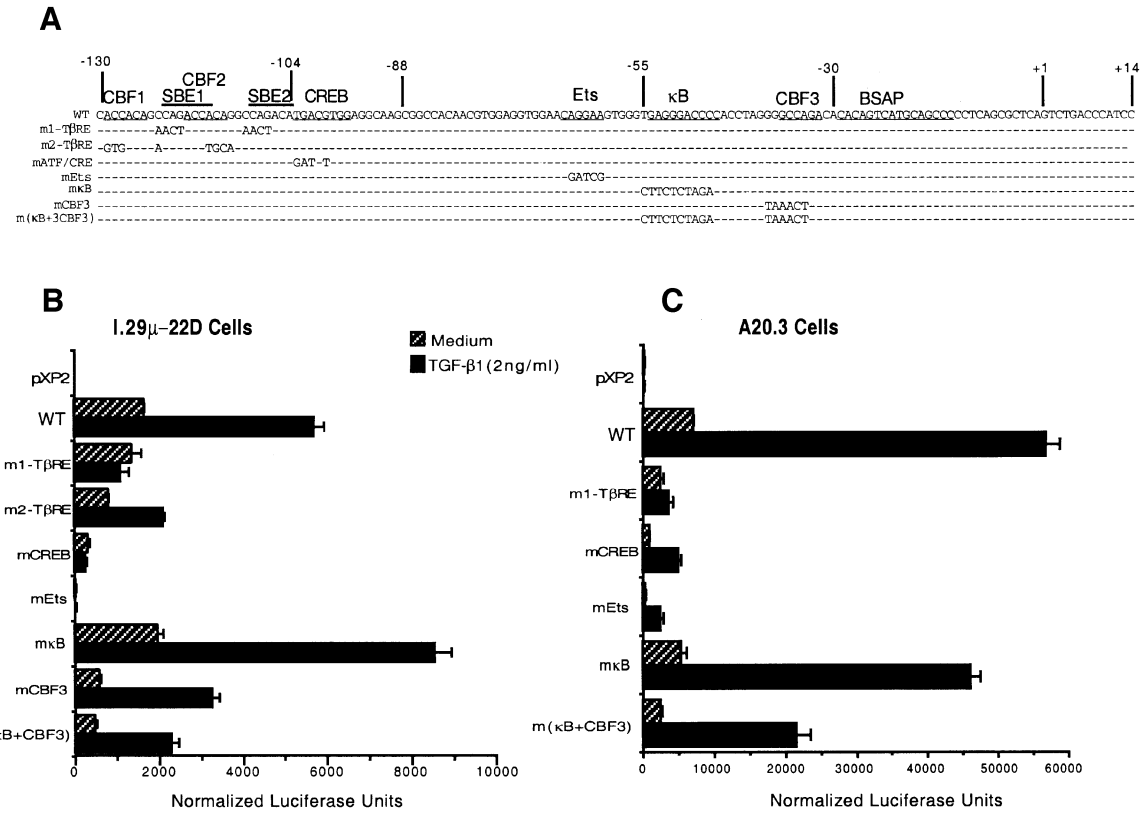


Fig. 1. Effects of mutations of the GL α promoter on Luc reporter gene expression in two mouse B cell lines. (A) Nucleotide sequence of the 5′ flank from –130 to +14 (except for m2-TβRE from –132 to +14) relative to the first transcription initiation site in the I/St mouse GL α gene (19). The previously-defined TβRE resides at –130/–104 and consists of a direct repeat with two binding sites for CBFα (CBF, which are underlined) and Smad proteins (SBE, indicated by over-lines) (see text for references). Below the wild-type sequence are indicated the introduced nucleotide substitutions. The wild-type and mutated DNA –130/+14 fragments of the GL α promoter (except m2-TβRE which has an additional GA sequence upstream of –132) were inserted upstream of the Luc reporter gene in pXP2 plasmid. (B and C) Luc activities of reporter plasmids transiently transfected into I.29μ (clone 22D) and A20.3 cells. TGF-β1 was added right after transfection (for both cell lines) and again at 7 h after transfection for I.29μ cells. Luc activity was assayed 24 h after transfection. Means + SE from at least three independent experiments are reported in (B, I.29μ cells) and (C, A20.3 cells) in light units after subtraction of background (no cell extract) of 200–300 light units. Variation of transfection efficiency among different plasmids was corrected by the activity of pSV2CAT which was co-transfected with the Luc plasmids. The promoterless plasmid pXP2 is the control vector.

Consensus transcription factor sites. Cre (CREB-binding site): 5′-GATTCGATGACGTCAGTACGGTG-3′; κB (NF-κB site): 5′ CAACGGCAGGGGAATTCCCCTCTCCTT 3′ (38); AP-1: 5′-AGCTTGGTGACTCATCCG-3′; CBFα: 5′-CGTATTAACCACAATACTCG-3′ (20).

Primers used for PCR. Lower case bold letters indicate mutated nucleotides; lower case plain text letters indicate nucleotides used for cloning that are not in the GL α promoter. Reverse PCR primer (+14/–7) 5′-cgcggatccTGGATGGGTCAGACTGAGCGC-3′; mEts, upper 5′-GGAAC**gatcg**GTGGGTGAGGGACC-3′; lower 5′-CCCAC**cgatc**GTTCCACCTCCACGTT-3′; mκB, upper 5′-TGGAACAGGAAGTGGGT**cttctctaga**ACCTAGGGGCCAGAC-3′; lower 5′-GTCTGGCCCCTAGGT**tctagagaag**ACCCACTTCCTGTTCCA-3′; mCBF3, upper 5′-CCCACCTAGG**taaacta**CACACAGTCATGCAG-3′; lower 5′-**agttta**CCTAGGTGGGGTCCC-3′; m(κB + CBF3) upper 5′-**tctaga**ACCTAGG**taaacta**CACACAGTCATGCA-3′; lower 5′-**agttta**CCTAGGT**tctagagaaG**ACCCACTTCCTGTTCCA-3′.

Single-stranded oligonucleotides were obtained from Operon Technologies (Alameda CA), DNA International (Woodlands TX), DNAgency (Aston, PA) or Ransom Hill Bioscience (Ramona, CA).

Plasmid constructs

Mutations of the GL α promoter (Fig. 1A) were created by PCR based on an overlap extension technique (39). PCR-amplified fragments carrying the desired mutations were then cloned into *Sma*I–*Bgl*II sites upstream of the luciferase (Luc) reporter gene in the pXP2 vector (40). Each construct was sequenced to confirm that the expected mutations had been introduced and that no extra mutations had been created. DNA sequencing was performed using the dideoxy chain termination method with Sequenase version 2.0 kits (US Biochemical, Cleveland, OH) or by automated sequencing using an Applied Biosystems sequencer and software (Foster City, CA).

Transfection

Transfection was performed by electroporation using Cell ZapII (Anderson Electronics, Brookline, MA) as described

(18). The GL α Luc reporter plasmids were co-transfected with one of three different internal control plasmids, depending on the experiment. pSV2CAT was used for I.29μ transfections shown in Fig. 1(B); pPGKβ-gal (41) or pCMVβ-gal (Stratagene, La Jolla CA) for all other transfections. Cells were incubated for the indicated times at 37°C and then assayed for Luc and chloramphenicol acetyltransferase (CAT) or β-galactosidase (β-gal) activity.

Expression plasmids

cDNAs for Smads 3 and 4, cloned in pcDNA3, were obtained from M. Kawabata (The Cancer Institute, Tokyo) (42). The expression plasmid for human AML2 (CBFα3) was obtained from S. Hiebert (St Jude Children's Research Hospital, Memphis, TN) (43). Mouse cDNA for Elf-1 cloned in pCDNA3 was obtained from M. Roussel (St Jude Children's Research Hospital) (44). This plasmid was used in the transient reporter assays. Human Elf-1 and PU.1 cDNAs used for *in vitro* transcription/translation in reticulocyte lysates were from L. Shapiro (St Jude Children's Research Hospital) (45). cDNA for mouse Fli-1, cloned in pGEM, was obtained from A. Bernstein (University of Toronto, Toronto, Canada) (46). The dominant-negative Ets expression plasmid was from E. Boulukos (Universite de Nice-Shophia Antipolis, Parc Valrose, France) (47). Rat CREB expression plasmid, pCMV2-CREB, was subcloned from the rat cDNA (48) by P. R. Dobner (University of Massachusetts Medical School).

Luc, CAT and β-gal assays

Luc and CAT assays were performed as described (37), and β-gal was assayed as described (49). The CAT activity was measured by a liquid scintillation counter after 4 h (for A20.3 cells) and 20 h (for I.29μ cells) incubation at 37°C, in the linear range of the assay.

Oligonucleotide probes for EMSA

To produce double-stranded oligonucleotides, complementary single-stranded oligonucleotides were annealed at 100 ng/μl each in 100 mM NaCl, 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA. Prior to annealing, the oligonucleotides were incubated at 95°C for 10 min to disrupt secondary structure, and then incubated at 10°C below the T_m for 1 h and slowly cooled to room temperature. The annealed oligonucleotides were ethanol precipitated and purified on a 12–15% polyacrylamide gel. Purified double-stranded oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. DNA segments corresponding to the GL α promoter from –130 to +14 were generated by PCR. They were gel-purified and end-labeled as described for the annealed oligonucleotides.

Preparation of nuclear extracts (NE)

NE for I.29μ, A20.3 and splenic B cells were prepared using the method of Schreiber *et al.* (50) as modified (37). In addition to several protease inhibitors, a phosphatase inhibitor Na_3VO_4 (Boehringer Mannheim, Indianapolis, IN) was added to buffer A and C at a final concentration of 1 mM. Protein concentration of the NE was determined using the Bradford assay (BioRad, Richmond, CA).

In vitro transcription and translation

For *in vitro* transcription and translation, hElf-1, hPU.1 and mFli-1 proteins were synthesized using the TNT-coupled reticulocyte lysate system (Promega, Madison, MI), according to the manufacturer's protocol. An aliquot of 1 μl of the resulting lysate was used for each EMSA incubation.

EMSA

DNA binding reactions were performed in 15 μl reaction volumes containing 0.1–1 ng (15,000–30,000 c.p.m.) end-labeled double-stranded DNA probe, 1–5 μg NE and 2–4 μg poly(dI–dC) (Pharmacia, Piscataway NJ). The final concentration of NaCl in each reaction mixture was adjusted to 80 mM by adding buffer C (50). The reaction mixtures were incubated at room temperature for 30 min and then loaded onto 4–6% native polyacrylamide gels. All gels were electrophoresed in re-circulating 0.5×TBE buffer at 100–150 V for 2–4 h. For supershift or competition experiments, antibody (or antiserum) (1 μl, except where indicated) or competitors were added to the entire mixture, except the probe which was added last.

Results

Effects of mutation of transcription factor binding sites on expression of the GL α promoter in two mouse B cell lines

Several transcription factors or their binding sites have been identified in the promoter for mouse GL α transcripts. To assess their relative importance for TGF-β1 induction and for the overall level of expression of the promoter, nucleotide substitution mutations were created in each of the known binding sites within the –130/+14 promoter segment (Fig. 1A). This segment drives TGF-β1-inducible activity of the Luc reporter gene in the promoterless pXP2 plasmid and is as active as the –128/+46 segment used in previous experiments (19). The plasmids were transiently transfected into I.29μ (the 22D subclone) and A20.3 B lymphoma cells. I.29μ (22D) cells express surface IgM and constitutively express GL α transcripts, which can be further induced by treatment with TGF-β1 (10). A20.3 cells express sIgG2a and have no detectable GL α transcripts when cultured with or without TGF-β1, although they have C_α genes (19). The plasmid pSV2CAT, which expresses the CAT gene under control of the SV40 enhancer/promoter, was co-transfected in these experiments as an internal control for variation in transfection efficiency.

In I.29μ cells, TGF-β1 increases Luc activity of the plasmid driven by the wild-type GL α promoter by 3.5-fold (Fig. 1B). This level of induction is consistent with previous results (19). These results are also in accord with the finding that TGF-β1 increases transcription of the endogenous GL α RNA in I.29μ cells by 5-fold in a nuclear run-on assay (10).

The DNA segment –130/–104 contains a direct repeat dimer that has two binding sites for Smad proteins (CAGAC, indicated as SBE1 and 2, Smad-binding element, in Fig. 1A) and two binding sites for CBFα (ACCACA, indicated as CBF1 and 2). This segment has been shown to be sufficient for conferring TGF-β1 responsiveness to a minimal *c-fos* promoter (19), and to be synergistically activated by Smads 3 and 4 and CBFα (18,22–24). Mutation of SBE 1 and 2 in construct m1-TβRE eliminates TGF-β1 induction but does not reduce

basal activity in I.29μ cells (Fig. 1A and B). Mutation of CBF1 and 2 (m2-TβRE) reduces TGF-β1 induction by ~30% and basal promoter activity by ~50%. Mutation of a downstream, weak CBFα binding site (CBF3) (18) reduces basal activity by 3-fold but has no effect on TGF-β1 induction. Consistent with published studies (18,24,25), mutation of the CREB-binding site eliminates induction by TGF-β1 and reduces basal promoter activity by 5-fold.

Surprisingly, mutation of a sequence element that matches a consensus NF-κB binding site (–54/–45) slightly increases promoter activity and has no effect on induction by TGF-β1. This site does bind NF-κB/p50 (see below). Two additional sets of mutations of the κB site were also tested in the reporter assay with similar results (data not shown). Mutation of both the κB element and the third CBFα binding site has an effect similar to mutation of the third CBFα site alone (Fig. 1B). Of all the sites in the promoter, the Ets consensus motif at –64/–61 (GGAA) appears to the most important for overall activity, since mutation of this site eliminates promoter activity, both basal expression and induction by TGF-β1.

The effects of the promoter mutations were similar but not identical when tested in A20.3 cells (Fig. 1C). TGF-β1 increases Luc activity of the wild-type promoter by 8-fold in A20.3 cells. Mutation of both SBEs (m1-TβRE) abolishes induction and reduces basal promoter activity by 60%. Mutation of the ATF/CRE or Ets site nearly eliminates promoter activity, although TGF-β1-induction is mostly retained. As in I.29μ cells, mutation of CBF3 reduces expression but has no effect on induction by TGF-β1 (data not shown) and mutation of the κB site has little effect on promoter activity. As in I.29μ cells, the effect of mutation of both the κB site and CBF3 is similar to the effect of mutation of CBF3. Unlike results in I.29μ cells, however, the m2-TβRE mutation does not significantly affect promoter activity (15% reduction for TGF-β1-induction and 10% reduction for basal activity compared with the wild-type promoter, data not shown). In both cell lines, TGF-β1 has no effect on the promoterless pXP2 plasmid.

Thus, the effects of mutation of some of the consensus elements differ somewhat between two cell lines, only one of which (I.29μ) can express endogenous GL α transcripts and switch to IgA. In both cell lines, the SBE are necessary for inducibility by TGF-β1. However, the CBFα-binding sites 1 and 2 differ in their importance in the two cell lines, being important for both basal and induced expression in I.29μ but not in A20.3 cells. This may be because TGF-β1 induces CBFα3 in I.29μ cells but not in A20.3 cells (18), although the constitutively expressed CBFα2 is equally abundant in these two cell lines (unpublished data). In I.29μ cells the ATF/CREB element is essential for induction by TGF-β1 and the Ets motif is essential for all promoter activity, whereas in A20.3 these elements are do not contribute to TGF-β1 inducibility although they are very important for overall promoter activity. Neither CREB nor Ets proteins have been reported to be inducible by TGF-β1. The data obtained in I.29μ cells are consistent with the hypothesis that CREB and the protein(s) binding at the Ets site interact directly, or indirectly, with the TGF-β1-inducible proteins, Smads and CBFα3, which bind at the TβRE.

To further establish the importance of the Ets site for activity of the promoter, we tested the effect of co-transfection of an expression plasmid for a dominant-negative version of Ets2 (47) along with the GL α promoter Luc reporter plasmid in A20.3 cells. As shown in Fig. 2, the dominant-negative Ets2 expression plasmid inhibits the basal expression and also TGF-β1 induction of the promoter in a dose-responsive manner. The recombinant dominant-negative Ets protein was shown to bind to the GL α Ets site in EMSA (data not shown). These data further indicate the importance of Ets family proteins for activity of the promoter.

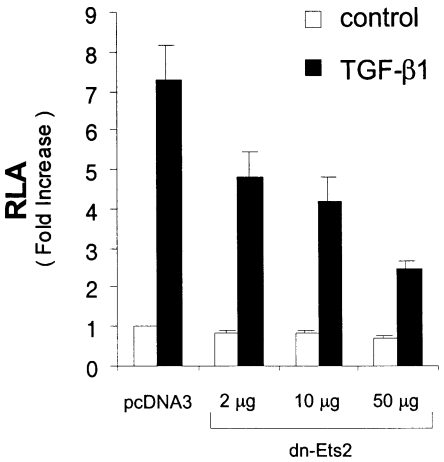


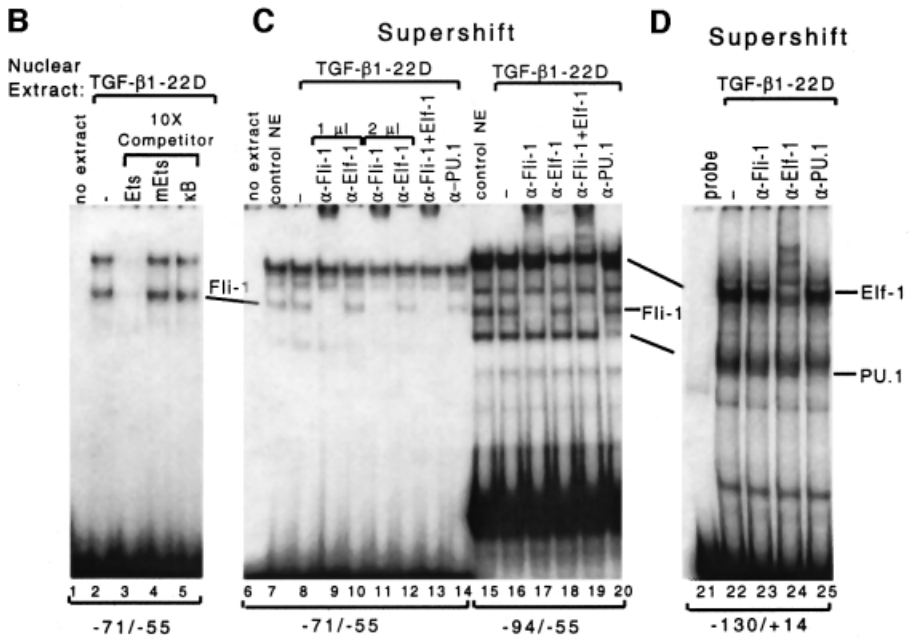
Fig. 2. Transient transfection assays demonstrate importance of an Ets protein on GL α promoter activity. Increasing doses of an expression plasmid encoding a dominant-negative Ets2 protein were co-transfected into A20.3 cells with 50 μg of the wild-type GL α-Luc reporter plasmid. TGF-β1 was added and cells were incubated 15 h. A pCMV-β-gal reporter plasmid was co-transfected for normalization for transfection efficiency. The data were obtained from three experiments and the error bars show the SEM.

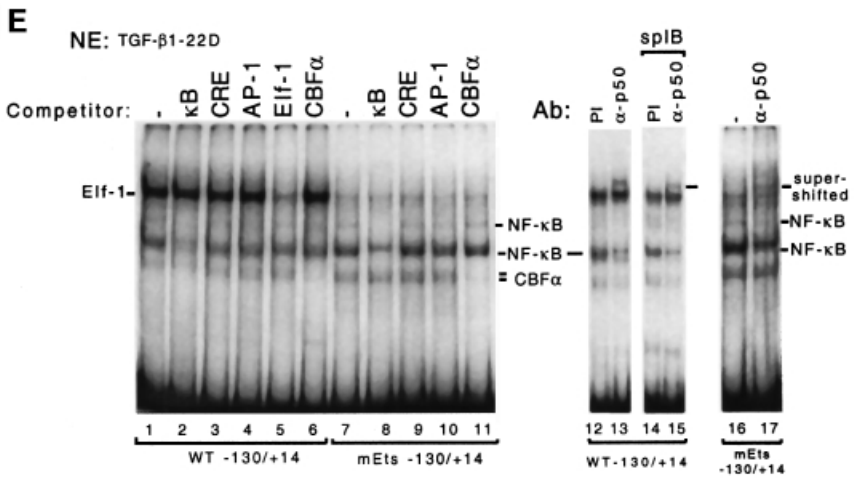
Identification of Ets proteins binding to the GL C_α promoter

Since a consensus Ets element is essential for expression of the GL α promoter, it was important to identify the Ets proteins which bind this site. In addition to the Ets site at –64/–61 indicated in Fig. 1(A), there is another consensus Ets site just upstream at –70/–67. There does not appear to be a counterpart for this upstream Ets site in the human and rabbit promoters (51,52). Using EMSA, we tested binding of nuclear proteins from TGF-β1-treated I.29μ cells to a 17 bp GL α DNA segment, –71/–55, that contains the two core Ets sites. Figure 3(A) presents the sequences of the probe with the two core Ets sites underlined. The core of the 5′ site is present in this probe, but since a functional Ets site includes 5 nucleotides upstream of the core sequence this site is incomplete. The 3′ Ets site is the site mutated in the reporter gene assays shown in Fig. 1. As shown in Fig. 3(B), two complexes are formed with this probe which can be competed by a 10-fold excess of the probe (Fig. 3B, lane 3) but not by double-stranded oligonucleotides containing a mutated Ets or wild-type κB site (Fig. 3B, lanes 4 and 5). Antibody supershift assays shown in Fig. 3(C, lanes 9, 11 and 13) reveal that the Ets protein Fli-1 forms the faster migrating complex. Components of the other complexes have not been identified and it is unknown if the two additional complexes

A Ets Site Probes

Optimum mElf-1 binding site	A(A/T)ACCGGAAGTGG
GL α-71/-55 (WT)	TGGAACAGGAAGTGGGT
GL α-71/-55 (mEts)	TGGAAC**gatcg**GTGGGT
GL α-83/-51 (WT)	ACAACGTGGAGGTGGAACAGGAAGTGGGTGAGG
GL α-83/-51 (m-61)	ACAACGTGGAGGTGGAACAGGA**t**GTGGGTGAGG
GL α-83/-51 (m-66)	ACAACGTGGAGGTGGAA**g**AGGAAGTGGGTGAGG
GL α-83/-51 (m-69/-68)	ACAACGTGGAGGTG**ct**ACAGGAAGTGGGTGAGG





formed with this probe, which are seen quite clearly in Fig. 3(C) but barely in Fig. 3(B), are specific. Antibodies to two other Ets proteins, Elf-1 and PU.1, do not affect the complexes formed with this probe (Fig. 3C, lanes 10, 12 and 14).

Surprisingly, when a DNA probe containing the full-length promoter used in the reporter assays is tested in the antibody supershift assays, we find that Fli-1 antibody does not affect the complexes, whereas Elf-1 antibody supershifts the predominant slowest migrating complex (Fig. 3D, lanes 21–24). The Ets site mutation used in the reporter gene assays shown in Fig. 1 eliminates Elf-1 binding (Fig. 3E, *cf*. lanes 1 and 7).

A DNA probe of intermediate length, containing the –94/–55 promoter segment, binds both Fli-1 and Elf-1, as both of these antibodies supershift complexes (Fig. 3C, lanes 17–19). In addition, PU.1 antibody inhibits the fastest migrating complex formed with the –94/–55 probe and inhibits a component of the fast-migrating major complex formed with the full-length promoter segment (Fig. 3C, lane 20 and Fig. 3D, lane 25). No other Ets antibody tested (Ets1/2, PEA3 and SpiB) affected complexes detected with any GL α probe (data not shown). TGF-β1 treatment has no effect on formation of the Ets complexes (Fig. 3C, *cf*. lanes 7, 8 and 15, 16; data not shown). NE from A20.3 cells gave similar results, except they have slightly lower levels of Elf-1 binding activity (data not shown).

These data indicate that the binding of Elf-1 and PU.1 to the GL α promoter requires, in addition to the –71/–55 segment, the flanking region. Neither of the two Ets consensus elements in the GL α promoter are optimal Elf-1 or PU.1 sites. An optimal Elf-1 site is shown in Fig. 3(A) (44,53). It has previously been shown that Elf-1 binds to and activates transcription at non-optimal sites by interacting with other proteins, including AP-1, HMG-I(Y) and NF-κB p50 and c-Rel (54–56). However, we have not found any binding sites for AP-1 or HMG-I(Y) in the GL α promoter and the NF-κB site is not required for promoter activity. Although Fli-1 binds to the small probe, it too may have a partner protein since the Fli-1 complex formed on the small probe migrates slightly more slowly than the complex formed with the –94/–55 probe (Fig. 3C, *cf*. lanes 14 and 15). Perhaps the partner proteins differ on these two probes.

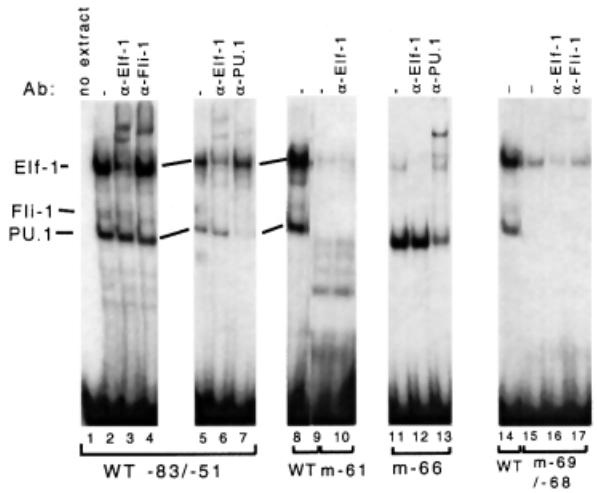


Fig. 4. EMSA using the –83/–51 segment from the GL α promoter to demonstrate the effects of nucleotide mutations on binding of Elf-1 and PU.1. Wild-type probes or probes with the indicated mutations (sequences shown in Fig. 3A) were incubated with 2 μg NE from untreated I.29μ cells in the absence or presence of 1 μl of the indicated antibody. All the lanes within each panel are from the same gel (and same exposure).

Sequences required for Elf-1 and PU.1 binding to the GL α promoter.

To attempt to identify the nucleotides required for binding of Elf-1 and PU.1 to the GL α promoter, several probes of different lengths were tested in EMSA using I.29μ NE. Addition of 4 nucleotides to the 5′ side of the Ets sites (–75/–55 segment) did not allow Elf-1 binding (data not shown). Addition of 4 nucleotides to the 3′ side (–75/–51) only allowed weak Elf-1 binding (data not shown). By testing several additional segments, we found that the nucleotides required for nearly optimal Elf-1 binding resided within the –83/–51 segment. This probe also binds PU.1. It also binds Fli-1, but to a lower extent than the –71/–55 probe (Fig. 4, lanes 1–7, and data not shown).

To further explore the sequence requirements for Elf-1 and PU.1 binding, we created single- or double-nucleotide mutations in the –83/–51 probe. Consistent with a previous report (57), mutation of a single A within the 3′ Ets site to T (m-61) eliminates Elf-1 binding to the –83/–51 probe (Fig. 3A, Fig. 4, lanes 9 and 10). This mutation also eliminates PU.1 binding, indicating that the Ets site at –64/–61 is required for both Elf-1 and PU.1 binding. Mutation of nucleotide –66 nearly eliminates Elf-1 binding but increases PU.1 binding,

Fig. 3. EMSA demonstrate that Elf-1 and PU.1 bind the intact GL α promoter. (A) Nucleotide sequences of the wild-type and mutant oligonucleotides used in EMSA and in reporter gene assays, and the optimal consensus Elf-1 binding sequence (44,53). (B) Competition EMSA using 2 μg NE from I.29μ cells treated with TGF-β1 for 14 h and the GL α –71/–55 segment as probe. The left-most lanes in (B)–(D) contain the probe alone. Competitor DNA segments are the wild-type –71/–55, a –71/–55 segment containing the same Ets mutation (mEts) as shown in Fig. 1(A) and a wild-type consensus κB (NF-κB binding site) oligonucleotide (see Methods). (C) Supershift EMSA using 2 μg NE from I.29μ cells, untreated or treated with TGF-β1 for 14 h incubated with the –71/–55 or –94/–55 promoter segment, as indicated. (D) Supershift EMSA using 2 μg of NE from TGF-β1-treated A20.3 cells and the wild-type –130/+14 full-length promoter segment used in the reporter gene assays shown in Fig. 1. (E) EMSA demonstrate that NF-κB/p50 binds the GL α promoter. Competition and supershift EMSA using the –130/+14 promoter segment with wild-type or mEts sites and 2 μg NE from TGF-β1-treated I.20μ cells. For competitions with the wild-type probe, the κB, CRE (CREB-binding site), Elf-1 and CBFα oligonucleotides were used at 500-fold excess, and the AP-1 oligonucleotide at 100-fold excess. For competitions with the mEts probe, the competitors were used at 50-fold excess except AP-1 was used at 90-fold excess. Supershift EMSA were performed with NE from TGF-β1-treated I.29μ cells or from and LPS + TGF-β1-treated splenic B cells, as indicated. An aliquot of 1 μl of antibody (Santa Cruz Biotechnology) was used in each reaction, unless indicated otherwise.

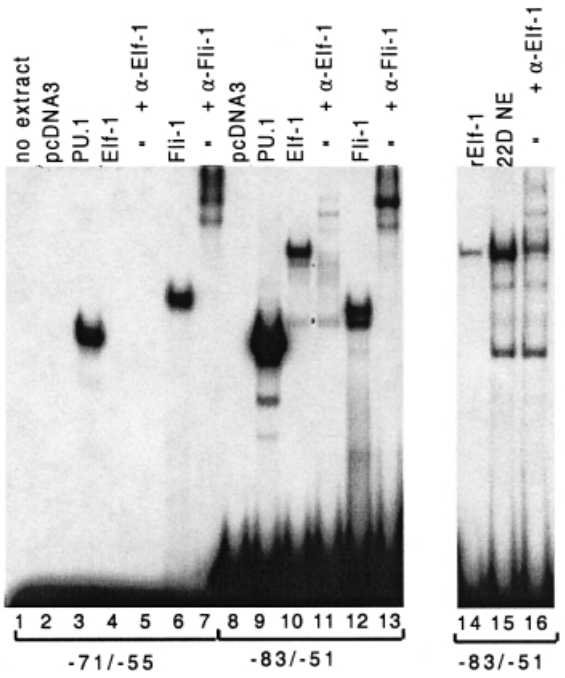


Fig. 5. Binding of *in vitro* translated Ets proteins to the GL α promoter segments. An aliquot of 1 μl of reticulocyte lysate obtained from *in vitro* translation of mRNA transcribed from the indicated plasmids was incubated with the indicated wild-type GL α probes. pcDNA3 corresponds to lysates programmed with the products from the empty expression plasmid. Lane 15 contains 1.5 μg NE from TGF-β1-treated I.29μ cells. An aliquot of 1 μl of the indicated antibodies was added in lanes 5, 7, 11, 13 and 16 to the same lysate or NE shown in the adjacent left lane, i.e. lane 5 contains lysate programmed with Elf-1 mRNA + antibody to Elf-1, etc.

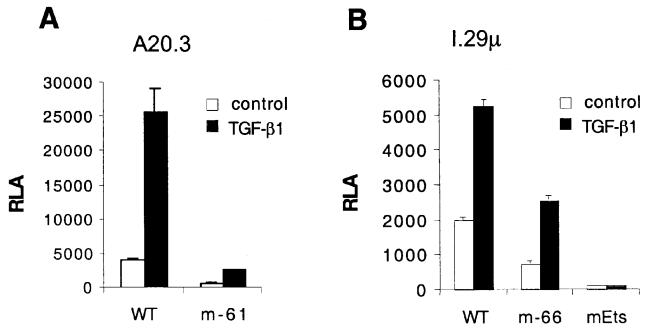


Fig. 6. Effects of mutations in the Ets sites of the GL α promoter on Luc reporter gene expression in (A) A20.3 and (B) I.29μ cells. The average results of two transfections are reported. The error bars show the range of the data. Figure 3(A) presents the sequences of all mutations studied. Luc reporter plasmids (50 μg) and β-gal control plasmids (30 μg) were transfected, cells were TGF-β1 treated, and Luc activity was assayed at 15 h in (A) and 22 h in (B).

suggesting that PU.1 binding does not depend on Elf-1 binding (Fig. 4, lanes 11–13). Mutation of nucleotides –69/–68 in the 5′ Ets site eliminates PU.1 binding and greatly decreases Elf-1 binding, suggesting that the 5′ Ets site is also important for binding of both these proteins (Fig. 4, lanes 15–17). To attempt to identify other proteins which bind the –83/–51 probe and might help Elf-1 binding, several sets of mutations were created in nucleotides flanking the two Ets sites. Although all the flanking nucleotides were mutated in various combinations in these experiments, these other mutations had only a small, or no, effect on Elf-1 binding (data not shown). It is possible that the particular mutations chosen do not prevent binding of a putative partner protein or that the partner protein binds non-specifically.

To examine whether binding of Elf-1 to the wild-type GL α promoter depends on B cell-specific proteins, we asked if individual recombinant Elf-1, Fli-1 and PU.1 obtained by *in vitro* translation using reticulocyte lysates would show the same binding specificity as found in B cell extracts. Reticulocyte lysates programmed by mRNA for the three different Ets proteins were used in EMSA with both the shorter and longer GL α Ets probes. Figure 5 shows that the short Ets probe, –71/–55, binds recombinant PU.1 and Fli-1 but fails to binds Elf-1 (Fig. 5, lanes 3–7), whereas the –83/–51 probe binds all three proteins (Fig. 5, lanes 8–13). Thus, recombinant Elf-1 binding retains the same specificity as it does in B cell NE. Also, although recombinant PU.1 does bind the short probe, it does not bind as well to the short probe as to the longer probe. Since the recombinant Elf-1 complex co-migrates with the complex formed with I.29μ NE (Fig. 5, lanes 14–16), it appears that if a partner protein is required, it is also present in reticulocyte extracts. Lysates programmed by the empty expression vector (pcDNA3) showed no binding to either probe. These data demonstrate that Elf-1 binding to the GL α promoter does not depend on PU.1 binding nor does PU.1 binding depend on Elf-1 binding. Furthermore, the role of the additional sequences surrounding the Ets sites is not specific to B cell lines. These data also indicate that although Elf-1 and Fli-1 bind in a reciprocal manner to the various Ets site probes, this is not simply due to Fli-1 competing with Elf-1, since when recombinant Elf-1 is tested individually, it shows the same binding specificity.

Effect of Elf-1 binding on activity of the GL α promoter

To provide further evidence for the importance of specific Ets proteins for activity of the GL α promoter, we tested the effects of two of the single nucleotide mutations in the reporter gene assay. Since the EMSA data shown in Fig. 4 indicate that the A at –61 is essential for binding of Elf-1 and PU.1 to the promoter, we tested the effect of this single mutation in A20.3 cells. As shown in Fig. 6(A), mutation of nucleotide –61 greatly reduces basal and TGF-β1-induced promoter activity, but not TGF-β1 inducibility, similar to the effect of mutation of the entire Ets core site (mEts) in A20.3 cells shown in Fig. 1(C). To attempt to determine if the promoter specifically requires Elf-1 for activity, we examined whether mutation –66, which nearly eliminates Elf-1 binding but increases PU.1 binding, would have an effect on transcriptional activity of the –130/+14 promoter in I.29μ cells. m-66 was found to reduce basal and TGF-β1-induced Luc activity by 2-fold (Fig. 6B). These results suggest that even when the binding of PU.1 is enhanced, PU.1 cannot completely substitute for Elf-1.

Activation of the GL α promoter by transcription factor expression plasmids

Since the EMSA data indicate that Elf-1 binds the GL α promoter, we wished to test if co-transfection of an expression

plasmid for Elf-1 would increase activity of the promoter in reporter gene assays. Expression plasmids for transcription factors known to be important for TGF-β1-induced expression of this promoter were also included to determine if Elf-1 might cooperate with them. As shown in Fig. 7(A), in I.29μ cells over-expression of CBFα3 or the combination of Smads 3 and 4 increases both the basal and TGF-β1-induced Luc activity relative to empty expression plasmids, although the addition of all three factors does not further enhance activity. Addition of Elf-1 to the combination of Smads 3 and 4 and CBFα3 does, however, further increase promoter activity.

We also tested the effect of over-expression of these transcription factors in A20.3 cells (Fig. 7B). It can be seen that, as reported previously (22) and unlike results obtained in I.29μ cells, Smads 3 and 4 synergize with CBFα3 to activate the promoter in A20.3 B lymphoma cells. However, in these cells Elf-1 does not further increase promoter activity. For unknown reasons, it is generally difficult to obtain increased transcriptional activity by over-expressing Ets proteins. This may be due to limiting amounts of a cooperating factor, since it has been reported that Elf-1 activity at non-optimal Elf-1 sites requires cooperation with partner proteins (53,55). Although we have tested PU.1, CREB, HMG-I(Y), NF-κB and p300, we have been unable to identify any proteins that synergize with Elf-1 to activate transcription from the promoter (data not shown).

Elf-1 binding is affected by LPS activation of splenic B cells

The above data suggest that Elf-1 or PU.1 binding is required for transcription of the GL α promoter in B cell lines. Therefore, it was important to determine if NE from splenic B cells activated to switch to IgA contain Elf-1 or PU.1 binding activity. Using the –130/+14 promoter segment in EMSA, we found that NE from resting splenic B cells do not show Elf-1 binding activity, but that after activation for 14 h with LPS alone, or with LPS + TGF-β1, binding activity is induced (Fig. 8, *cf.* lanes 2, 3 and 5, 6; data not shown). PU.1 binding to the promoter was not detected. Induction of Elf-1 activity may be one of the roles of LPS in activating class switching in splenic B cells. However, LPS has additional roles since it is required for induction of CSR in I.29μ cells (10), although it is not required for Elf-1 binding activity in these cells (Fig. 8, lanes 8 and 11).

NF-κB binds the promoter but has little effect on its activity

It has been reported that NF-κB/p50-deficient B cells cannot undergo class switching to certain isotypes when activated in culture or by immunization (32,33). NF-κB has been shown to be important for expression of GL transcripts for some isotypes and this may be the explanation for the inability of p50-deficient B cells to switch to these isotypes. Although it has been shown that p50-deficient B cells switch poorly to IgA, they express normal levels of GL α transcripts. Consistent with these published data, mutations within the NF-κB consensus binding element in the GL α promoter do not reduce its activity in the reporter assay (Fig. 1B and C).

Because of this result it was important to demonstrate that the consensus κB element actually binds NF-κB. In the EMSA shown in Fig. 3(E), using the –130/+14 probe, we demonstrate that a consensus κB site competes a component of the fastest-migrating predominant complex (Fig. 3E, lane 2), and also competes with this complex and an additional slower-migrating complex detected using the mEts site probe

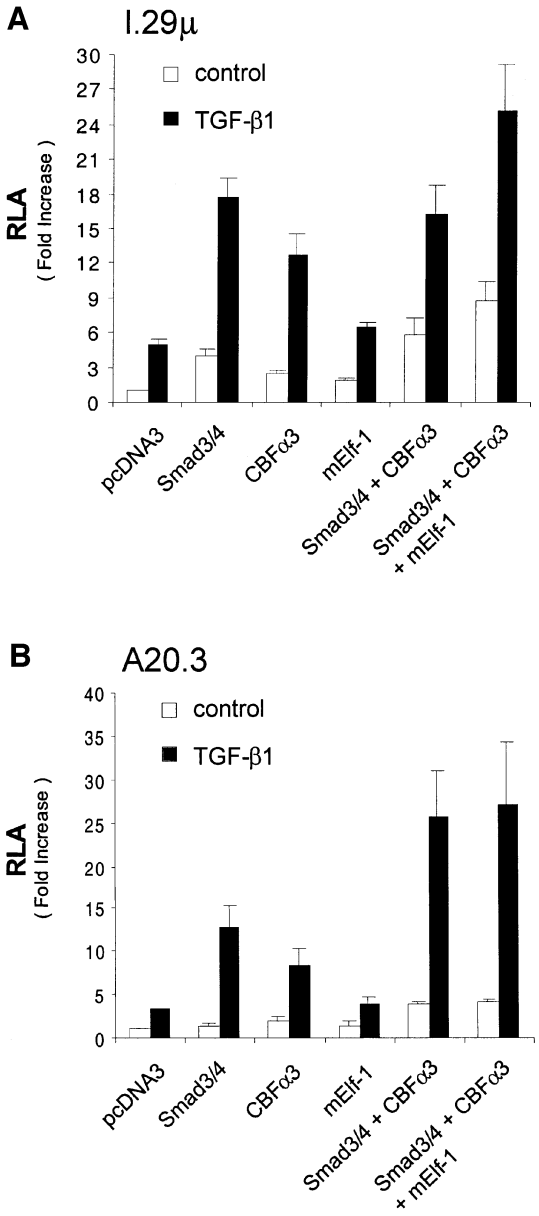


Fig. 7. Effect of co-transfection of expression plasmids for Ets family proteins, Smads 3, 4 and CBFα3 on transcriptional activity of the GL α reporter plasmid. (A) Luc activities of the GL α –130/+14 reporter plasmid (50 μg) transiently transfected into I.29μ cells along with 4 μg of expression plasmids for the indicated transcription factors. TGF-β1 was added right after transfection at 2 ng/ml. Luc activity was assayed 12 h after transfection. Means + SEM from three independent experiments are reported in light units after subtraction of background (no cell extract) of 200–300 light units. Variation of transfection efficiency among different plasmids was corrected by the activity of β-gal expressed from co-transfected pCMV-β-gal. (B) Luc activities from the identical experiment performed in A20.3 cells (n = 4), except that 2 μg of expression plasmids was used and cells were harvested for the Luc assay at 16 h after transfection.

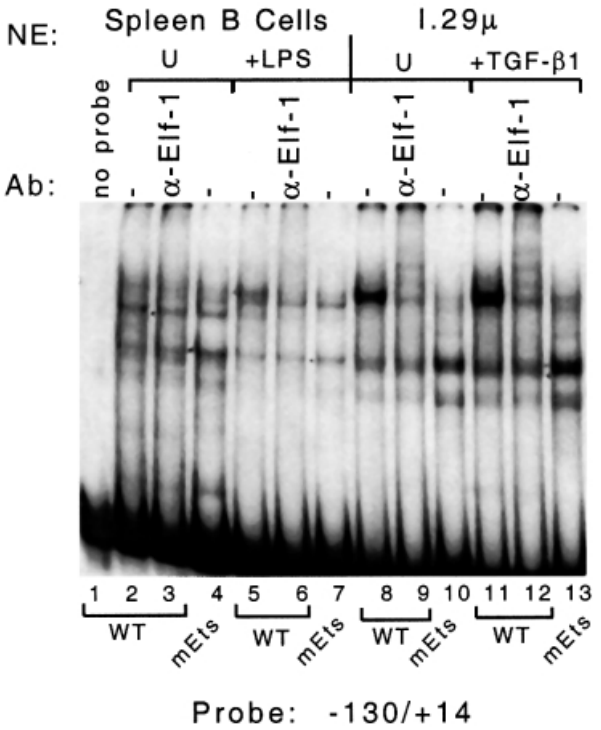


Fig. 8. EMSA demonstrating that Elf-1 binding activity is induced in splenic B cells by LPS treatment. Wild-type and mEts GL α –130/+14 segments were incubated with 4 μg NE from splenic B cells, untreated or treated with LPS for 14 h, or 2 μg I.29μ NE, untreated or treated with TGF-β1 for 14 h. Antibody to Elf-1 (1 μl) was added as indicated.

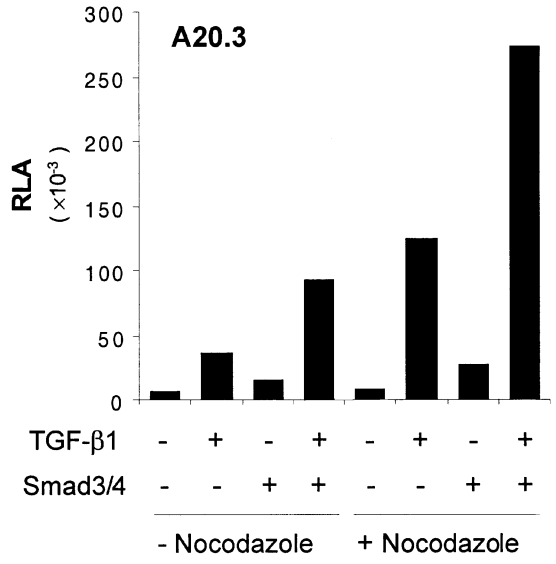


Fig. 9. Nocodazole induces Luc activity expressed by the wild-type GL α reporter plasmid in A20.3 cells. Immediately after transfection with 50 μg of the wild-type –130/+14 α reporter plasmid ± 15 μg expression plasmids for Smad3 and 4, A20.3 cells were treated with nocodazole (5 μg/ml) in the presence or absence of TGF-β1. After 16 h, cells were harvested and Luc activity was determined. The activity was normalized for transfection efficiency with the co-transfected pCMV-β-gal plasmid. These data are from one experiment but nocodazole had the same stimulatory effect in several other experiments.

(Fig. 3E, lane 8). Presumably, the faint upper NF-κB complex also binds the wild-type promoter segment but cannot be detected due to the background at this position in the lane. To determine whether these two complexes contain NF-κB/p50, supershift assays were performed, as shown in Fig. 3(E, lanes 12–17). Using the –130/+14 probe, anti-p50 supershifts a component of the fastest-migrating major component detected with NE from I.29μ cells (Fig. 3E, lane 13) and also from LPS + TGF-β1-treated splenic B cells (Fig. 3E, lane 15). Using the mEts probe in supershift experiments with antibody to NF-κB p50, we find that both complexes are supershifted (Fig. 3E, lane 17).

We reasoned it was possible the κB mutations do not affect activity of the GL α reporter plasmid because the amount of NF-κB is too low in the B cell lines to activate the promoter. To examine this possibility, we tested the effect of over-expression of several NF-κB proteins in co-transfection reporter gene assays. None of the expression plasmids tested, those encoding p50 alone, or RelA, c-Rel or RelB in combination with p50 stimulated GL α promoter activity in I.29μ cells (data not shown). We conclude that although NF-κB can bind the GL α promoter, it does not have a significant effect in transient reporter gene assays.

Nocodazole treatment increases GL α promoter activity

Smads 2, 3 and 4 have recently been shown to be associated with microtubules in the cytoplasm of epithelial, endothelial and HeLa cells lines (58). TGF-β1 treatment of an epithelial cell line results in the release of Smads from microtubules, and subsequent phosphorylation and migration to the nucleus where they activate transcription. Dong *et al.* (58) were able to mimic and enhance the effect of TGF-β1 on transcription by treatment of the epithelial cell line with nocodazole, which disrupts microtubules. We were interested to learn if Smad proteins might also be associated with microtubules in B cell lines, so we tested whether nocodazole could stimulate transcription driven by the GL α promoter in a B cell line. Consistent with the results in epithelial cells, we found that nocodazole increases activity of the promoter in A20.3 B cells by 2- to 3-fold, in the presence or absence of TGF-β1, and also when Smads 3 and 4 are over-expressed (Fig. 9). These data suggest that in the absence of TGF-β1, transiently transfected Smad proteins associate with microtubules in B cell lines, e.g. A20.3, and that TGF-β1 disrupts this association.

Discussion

An Ets site is essential for expression of the GL α promoter

In this study we compared the effect of mutation of several transcription factor binding sites on expression and TGF-β1 induction of the mouse GL α promoter. We demonstrated that an Ets site is essential for expression of this promoter and also for its inducibility by TGF-β1 in I.29μ cells. This site binds Elf-1 and PU.1 in the context of the full-length promoter used in transfection experiments. We also demonstrated that one of the several CBFα binding sites located 3′ to the direct

repeats of the TβRE contributes to expression of the promoter in reporter assays, but that a nearby NF-κB-binding site does not. Finally we showed that nocodazole, an inhibitor of microtubule formation, stimulates promoter activity. As previously shown for endothelial and epithelial cell lines (58), this last finding suggests that Smad proteins are sequestered in the cytoplasm via an association with microtubules in B cell lines.

Ets proteins have been implicated in regulation of genes during a variety of biological processes, including growth control, transformation, lymphocyte differentiation and developmental programs in many organisms. About 20 proteins have been identified that have the conserved *ets* domain and which bind the consensus Ets motif, found in both enhancer and core promoter regions. Ets family proteins bind to DNA as a monomer and generally bind cooperatively with other transcription factors, e.g. AP-1 (54,56,59–61), BSAP (62), Pip (NF-EM5) (63,64) MafB (65), Sp1 (66), serum response factor (67) and CBFα (68,69). Elf-1 and NF-κB have been shown to interact and to often bind adjacent sites on promoters (30,55,70). However, although the Ets and κB sites in the GL α promoter are also near each other, they do not appear to cooperate in that the Ets site is essential and the κB site appears to have no role in transcriptional activity. By contrast, CBFα, another protein known to interact with Ets proteins, is important for TGF-β1-induced activity of the GL α promoter.

Consistent with a requirement for cooperative binding with another transcription factor, the Ets sites of the GL α promoter bind Elf-1 and PU.1 when the probe used for EMSA contains at least 13 nucleotides 5′ and 10 nucleotides 3′ to the core Ets elements (GGAA). However, if a short fragment containing 7 nucleotides upstream and 6 nucleotides downstream of the 3′ Ets core is used as a probe, Fli-1 binds but not Elf-1 or PU.1. These data indicate that nucleotides in addition to the 3′ Ets site are required for Elf-1 and PU.1 binding. Recombinant Elf-1 and PU.1 tested individually in EMSA retain the same binding specificity, indicating that the differential binding is not due to competition among these Ets proteins for the same binding sites. From these data we conclude that Elf-1 and PU.1 are likely to be the proteins which bind *in vivo*. The binding differences may be due to the requirement for additional binding sites for different partner binding proteins. However, we have tested the effect of mutating several different nucleotides in the flanking regions of the –83/–51 Ets probe on binding by Elf-1 and PU.1, and find that the only mutations that significantly reduce binding are those within or between the two Ets sites (Fig. 4 and data not shown). Therefore, we have been unable to obtain direct data implicating interactions between these Ets proteins and other transcription factor families in the regulation of the GL α promoter.

Although we obtained no direct evidence of protein–protein interaction, our reporter gene assays suggest that Ets proteins which bind the GL α promoter interact with other factors to regulate the TGF-β1-inducible and constitutive activity of the promoter. Mutation of the more 3′ Ets site not only eliminates basal expression but also eliminates TGF-β1 inducibility of the GL α promoter in I.29μ cells. In addition, over-expression of a dominant-negative form of Ets 2 only inhibits basal activity of the promoter by 30%, but inhibits TGF-β1 inducibility by 50% in A20.3 cells. Furthermore, extensive gel shift and Western blotting analyses failed to find evidence that TGF-β1 induces Ets proteins in the two B cell lines or in splenic B cells (Fig. 3 and data not shown). Because of these data, we hypothesize that Ets proteins, presumably Elf-1 and PU.1, may interact with complexes involving TGF-β1-induced factors and thereby contribute to TGF-β1 induction of the promoter. Candidates for this interaction are the TGF-β1 inducible factors that bind the GL α promoter, i.e. Smads 3 and 4 and CBFα3. This complex might also involve CREB, since this factor also contributes to TGF-β1 induction of the promoter (Fig. 1B) (19,25,26). Perhaps the reason we have been unable to demonstrate this interaction in co-transfection reporter gene assays is due to limiting amounts of another factor.

Elf-1 is present in two B cell lines and is inducible in splenic B cells treated with the B cell mitogen LPS

Although the binding activity of Elf-1 is constitutive in I.29μ and A20.3 cells, we found that it is inducible by LPS in splenic B cells. LPS has been shown to activate p38 MAP, ERK and JNK kinases (71,72). Elf-1 binding activity has been shown to be dependent upon serine phosphorylation and many of the phosphorylated serine residues match consensus MAP kinase sites (30). PU.1 can also be phosphorylated on serine by JNK1 and by casein kinase II *in vitro*, and is phosphorylated in B cells. However, no functional significance for PU.1 phosphorylation has been demonstrated (30).

In addition to being regulated by phosphorylation, Elf-1 activity is regulated by binding to the cell-cycle regulator Rb. Elf-1 binds to the hypophosphorylated form of Rb protein, but not the phosphorylated form (73). During the transition from the G_1 to S phase of the cell cycle, Rb becomes phosphorylated, and the DNA binding and transcriptional activities of Elf-1 are induced in peripheral blood T cells. Since LPS induces kinase activity and B cell proliferation, it is possible that LPS induces Elf-1 activity by two mechanisms, by serine phosphorylation and by dissociation from Rb. CSR in splenic B cells requires cell proliferation (74) and during this proliferation Elf-1 may be activated. Furthermore, IgA switching *in vivo* occurs in the highly stimulatory environment of the intestinal lymph tissue called Peyer's patches, in which B cells are rapidly proliferating (75,76). The bacterial products endotoxin, non-methylated CpG-containing DNA and other bacterial cell wall components within this environment induce B cell activation and proliferation (77,78). Our data suggest that induction of Elf-1 binding after activation of B cells during an immune response is important for GL α transcription and subsequent switching to IgA.

NF-κB binds to the GL α promoter but has no effect on its activity in transient transfection reporter assays

The finding that mutation of the NF-κB binding site has no effect on activity of the GL α promoter in reporter gene assays was surprising, although this finding is consistent with results showing that p50-deficient B cells synthesize normal levels of GL α transcripts (33). NF-κB sites have been found in the promoters for three other mouse IgH GL transcripts: ε (29,79), γ1 (37) and γ3 (80). These κB sites have been shown to be essential for expression of the GL ε and γ1 promoters, and for their induction by IL-4 and/or by CD40L (29,37,79,81).

The composition of the NF-κB complexes binding a κB site affects its function (37,38,82–84). For the mouse GL γ1 promoter, RelA and RelB/p50 heterodimers were found to be activating, whereas p50 homodimers and c-Rel-p50 heterodimers were found to be inhibitory (84). Although we have been unable to detect any NF-κB protein besides p50 in the two complexes that bind the GL α promoter, the migration of the complexes is consistent with that of heterodimers of RelA, RelB or c-Rel with p50 (upper complex) and p50 homodimers (lower complex). It is possible that the κB site plays a role in a process other than transcription of the GL α promoter since switching to IgA in p50-deficient B cells is greatly reduced (32,33).

In conclusion, GL α transcription in the mouse B cell line I.29μ is activated by a combination of transcription factors inducible by TGF-β1 which interact with factors constitutively expressed in this B cell line. The most important of the constitutive factors is one or more Ets protein, most likely Elf-1 and possibly PU.1. However, in resting splenic B cells, neither Elf-1 nor PU.1 binding activity is detectable, although Elf-1 binding is inducible by LPS, and the combination of LPS and TGF-β1 induces GL α transcripts (9). Future studies will need to address the mechanism of Elf-1 induction in B cells and whether Elf-1 directly interacts with transcription factors induced by TGF-β1.

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Abbreviations

β-gal	β-galactosidase
CAT	chloramphenicol acetyltransferase
CBFα	core binding factor
EMSA	electrophoretic mobility shift assay
GL	germline
LPS	lipopolysaccharide
Luc	luciferase
NE	nuclear extracts
SBE	Smad-binding element
TβRE	TGF-β1-responsive element
TGF	transforming growth factor

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