Promoter Regulatory Elements and DNase I-hypersensitive Sites Involved in Serglycin Proteoglycan Gene Expression in Human Erythroleukemia, CHRF 288-11, and HL-60 Cells*

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Barbara P. Schick‡, Irina Petrushina, Kristin C. Brodbeck, and Patria Castronuevo

From the Cardeza Foundation for Hematologic Research, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

We have compared regulation of the serglycin gene in human erythroleukemia (HEL) and CHRF 288-11 cells, which have megakaryocytic characteristics, with promyelocytic HL-60 cells. Deletion constructs were prepared from the region −1125/+42 to −20/+42, and putative regulatory sites were mutated. In all three cell lines, the two major regulatory elements for constitutive expression were the (−80)/ets site and the cyclic AMP response element (CRE) half-site at −70. A protein from HEL and CHRF, but not HL60, nuclear extracts bound to the (−80)/ets site. Another protein from all three cell lines bound to the (−70)/CRE. Phorbol 12-myristate 13-acetate (PMA) and dibutyryl cyclic AMP (dbcAMP) increased expression of the reporter in HEL cells 2.5-3- and 4.5-fold, respectively, from all constructs except those with (−70)/CRE mutations. PMA virtually eliminated expression of serglycin mRNA and promoter constructs, but dbcAMP increased expression in HL60 cells. The effects of PMA and dbcAMP on promoter expression correlated with mRNA expression. The strengths of two DNase I-hypersensitive sites in the 5′-flanking region and the first intron in all three cells correlated with relative endogenous serglycin mRNA expression. An additional DNase I-hypersensitive site in HL60 DNA in the first intron may be related to the high serglycin expression in HL60 relative to HEL or CHRF cells.

The serglycin proteoglycan is synthesized by a number of hematopoietic cells, including platelets (1) and their parent megakaryocytes (2), granulocytes, macrophages, and lymphocytes (3–6), mast cells (4), and a large number of hematopoietic tumor cell lines (3, 7–10). Mature erythrocytes do not contain proteoglycans. We have recently found that serglycin is a major component of endothelial (11), murine uterine mesometrial decidual (12), and murine embryonic stem cell proteoglycans. Serglycin is thought to be critical for packaging and storage of various proteins in and secretion from hematopoietic cell granules and for modulating their activity. Serglycin binds to and stabilizes the chymases in the protease-containing secretory granules in mast cells (4, 13) and binds to granule-associated cytokines or chemokines such as MIP-1α and platelet factor 4 (14). Serglycin can bind to a number of matrix proteins such as fibronectin and collagen (9, 15, 16) and thus can influence cell/matrix interactions (16). We have reported that the serglycin proteoglycan of platelets of Wistar-Furth rats, which have a severe defect in α granule structure and protein content, has abnormally shortened glycosaminoglycan chains (15). Mice with a gene deletion of an enzyme required for heparin biosynthesis have severe defects in mast cell granules because of the defect in the heparin serglycin glycosaminoglycan chains (17, 18). Serglycins from other hematopoietic cells, endothelial cells, and decidua contain primarily chondroitin sulfate (2, 4, 8, 9, 19). The overall structure of the intact proteoglycan, rather than just the nature of the glycosaminoglycan chains, appears to govern the interactions of the molecule with other proteins such as collagen, fibronectin (9, 15), and CD44 (20, 21), suggesting that the core protein is important for organizing these interactions. The intact serglycin proteoglycan could play important modulatory roles in such diverse processes as hematopoiesis, inflammation, and coagulation. It is therefore of interest to understand how the synthesis of this proteoglycan is regulated in various types of hematopoietic cells.

Little is known about the mechanisms that regulate either constitutive or stimulated expression of the serglycin gene. Avraham et al. (22) transfected a series of 10 deletion constructs within the −500/+24 region of the murine serglycin promoter into rat basophilic leukemia cells and identified several broad positive and negative regulatory regions of the murine serglycin promoter. A putative regulatory element was suggested by loss of nearly all activity upon truncation at −81 in the middle of an ets element, but this site was not characterized.

A schematic diagram of the possible regulatory sites of the human serglycin gene is shown in Fig. 1 and is based on the sequence information provided by Humphries et al. (7). The human (7, 23) and murine (24, 25) serglycin genes are 96% conserved from −1 to −119 and have considerable homology for an additional 200 bases. A long purine-rich tract (dA80 in the human gene) is present at −580 in the human gene, and a similar region is present at −624 in the murine gene (25). Examination of the 5′-flanking sequences of the human and mouse serglycin genes reveals a number of potential elements that could regulate the expression of this gene. The conserved −1/−119 region includes a glucocorticoid response element at −64, a cyclic AMP response element (CRE)2 half-site at −70,
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and an ets site, CAGGAA, at −80. The CRE site was of interest because of its important role in the regulation of several hematopoietic genes (26, 27). The ets site was of interest because of the loss of promoter activity when this site in the murine gene was truncated (22). A reverse GATA-binding site (TTATC) at −357, present in the human but not in the mouse gene, is identical to a regulatory site of the megakaryocyte glycoprotein (GP) IIb gene (28–31). A GATA site either alone or in combination with an ets site is thought to regulate a number of genes that are restricted in expression to megakaryocytes or eryth-}

EMSA, electrophoretic mobility shift assay; GP, glycoprotein; HEL, human erythroleukemia; hGH, human growth hormone; kb, base pair(s); MOPS, 4-morpholino propane-sulfonic acid; kb, kilo base(s).
and hybridization were carried out at 50 °C as described (45) using a
buffer containing 50 mM potassium chloride, 10 mM sodium chloride,
1 mM magnesium chloride, and 0.1% sodium dodecyl sulfate. Signals
were detected by autoradiography.

**Electrophoretic Mobility Shift Assays**

Nuclear extracts were prepared essentially by the method of Dignam
et al. (46). The integrity of the extracts was tested by performing
EMSAs as described below with consensus oligonucleotides for univer-
sal transcription factors such as TFIID, Sp1, and AP-1 (Stratagene, La
Jolla, CA).

Oligonucleotides for the EMSAs were generally 24-mer probe said on
a nucleotide synthesizer at our institutional nucleic acid facility. The
sequences listed below and their complementary strands were hybridized,
and efficiency of hybridization was monitored on 4% agarose gels
run in Tris acetate/EDTA buffer. The double-stranded probes were
end-labeled with [γ-32P]ATP (New England Nuclear) and poly-nucleo-
tide kinase (Life Technologies, Inc.) and purified on Sephadex G-25
columns. The forward sequences, mutations, and other reagents for
in vitro transcription were a 21-TG fragment derived by reverse trans-
scriptase-PCR from the CREB consensus binding site. Primers were
5'-ATAACGATGCGTTGCTCTG and 5'-GGCTGATCCAGAGTAGTC,
spanning nucleotides 73–344, based on the cDNA sequence published by
Nicodemos et al. (23). The probe identified the expected
1.3-kb band on Northern blots.

**Southern Blot Analysis**—DNAs (5 μg/sample) were digested with
HindIII, and the DNA fragments were separated in 1% agarose gels by
electrophoresis, transferred to nylon membranes (Hybond N+; Amer-
shammer Biosciences), and hybridized at 65 °C overnight in a solu-
tion containing 50 mM Tris-HCl, pH 7.5, 1 mM NaCl, 1% SDS, 10%
xtran sulfate (Fisher), 0.05 mg/ml salmon sperm DNA, and the H106
probe radiolabeled by the random priming method (RadPrime DNA
Labeling System by Life Technologies, Inc.). The membrane was
washed once with 0.2x saline sodium citrate at room temperature for
5 min, followed by 0.5x saline sodium citrate at 65 °C for 2–3 min. The
probe abutting the HindIII restriction site in intron 1 of serglycin gene
was 106 base pairs (H106), generated using as forward primer nucleo-
tides 1234–1325 (5'-TCATTTTGGACGCTGCC-3') and as reverse
primer nucleotides 3232–3233 (5'-CCACCTGGCAGTGGTAG-3') based
on the numbering sequence of Humphries et al. (7). The sequence of
the probe was confirmed by automated sequencing, using the above
forward and reverse primers.

**Reverse Transcriptase-PCR Assays**—RNA from human HaCaT ker-
tinoocyte cells was extracted with Trizol reagent (Life Technologies,
Inc.). Reverse transcriptase-PCR was performed as described previ-
ously (11). Actin and GAPDH primers were used as positive controls.
The forward and reverse primers for human serglycin were as described
(11).

**RESULTS**

**Transfections of HEL, CHRF-288-11, and HL-60 Cells with the Human Serglycin Promoter Constructs**

The results of the transfection experiments are shown in Fig. 1.
Fig. 1o delineates the scheme we designed for sequential
removal of putative active sites in the deletion constructs. The
data shown in Fig. 1 (b and c) are from seven experiments on
each cell line that were all performed using a single batch of
each vector, and the entire series of vectors was used in each experiment.
Similar results were obtained in other experiments when other batches of the vectors were used. Fig. 1b shows the data from our deletion constructs, and Fig. 1c shows data from the mutagenesis of some of the putative regulatory sites.
The data for the deletion constructs in Fig. 1b are expressed relative to the —543/-42 construct, which served as the template for most of the mutations. The mutagenesis data in Fig. 1c are expressed relative to the —543/-42 constructs used within the same experiment. In separate experiments in which the external PCMvHGH control or the PCMv-SEAP or pSV40SEAP internal control was used, the pattern of utilization was consistent with that shown here.

The most important positive regulatory elements common to all three cell lines appeared to be between —49 and —54, because expression was most greatly reduced when this region was deleted (Fig. 1b). This region contains an ets site at —50, a CRE half-site at —70, and a glucocorticoid response element at
—64. There were some other interesting differences in promoter utilization both between the two megakaryocytic cell lines and between these and the HL-60 cells (Fig. 1b). Because the same preparations of vectors were used with all the cell lines for these experiments, we believe that the cell lines indeed utilize these constructs differently. For example, in the HEL
cells, the expression from the —543/-42 construct was always
25–50% less than that of the —344/-42 using several different vector preparations. In contrast, in experiments performed at the same time with the CHRF cells, the —344/-42 vector always gave somewhat less expression than did the —543/-42.
Deletion of the —543/-344 region removes the (~922)E-box,
(~484) partial CRE, and (~357) reverse GATA sites. Removal
Fig. 1. Analysis of the human serglycin promoter by deletion constructs and mutagenesis. a, schematic representation of putative regulatory sites in the human serglycin promoter, and the organization of these sites in the deletion constructs. b, promoter analysis of the serglycin gene in HEL, CHRF, and HL-60 cells. The deletion constructs shown in panel a were used. The experiment was performed seven times using the same batch of vectors for all three cell lines, but similar results were found with different batches of vectors in other experiments with different batches of vectors. In additional experiments (not shown) the \(-20/+42\) construct was used. This construct exhibited about half the activity of the \(-54/+42\). The data were calculated relative to the \(-543\) construct. The same profile was obtained when the data were calculated relative to the pCMVGH external control for each experiment or whether the internal control pCMVSEAP was used. c, the activity of the mutations performed in the context of the \(-543\) or the \(-284\) construct. The data for one GATA mutation (m1) are shown; the second mutation gave essentially the same results.
of only the region from −543 to −504 did not reduce promoter activity significantly in either the HEL or CHRF cells but reduced activity by about one-third in the HL-60 cells. Another striking difference was that in HEL cells, truncation of the −238 construct to −89 resulted in a 50% loss of activity, but there was no significant change in activity in the CHRF cells and HL-60 cells. These differences might be due to the use of a Sp1-like site, CCCACCC, in the deleted region by HEL cells. We have not evaluated specifically the effect of this site. One aspect unique to HL-60 cells was the substantial increase of activity in the −284 construct compared with −344 and −89. We have no explanation for this finding.

Another characteristic common to the three cell lines was the low activity of the three longest constructs. We have seen the same effect with the murine promotor in various murine cell lines,3 using the −1248/+24 sequence of the promotor described by Angerth et al. (25). The genes have in common a long poly(dA) stretch and a purine stretch, and the activity of the deletion constructs from both genes increases when these regions are removed. An analogous poly(dT) region is involved in regulation of activity of the rat and human PF4 genes (44, 50, 51). Specific proteins bind to the poly(dT) tract of the human PF4 promotor (44). Such long poly(dA) or poly(dT) stretches can interfere with nucleosome formation (52).

Mutation of either the ets or CRE sites greatly reduced the activity of the constructs in all three cell lines, and the double mutation reduced activity nearly to background levels (Fig. 1). The effect of the CRE mutation was greater than that of the ets mutation in the −284/+42 construct, and the effect of the ets mutation was greater than that of the CRE mutation in the −543/+42 construct in all three cell lines, but in all three cell lines the amount of inhibition resulting from mutation of the ets site plus the CRE site in the single mutations, as well as the effect of the double ets/CRE mutation, was close to 100%. The reason for the different relative behaviors of the mutated elements in the different length constructs is not understood. Two different mutations of the GATA site failed to alter the activity of the serglycin promotor in any of the cell lines (Fig. 1c). Mutation of the −522CATCTG E-box resulted in significantly reduced expression, about 50% in HEL and HL60 and 25% in CHRF cells (Fig. 1c), in contrast to the lack of effect of the loss of this element in the deletion constructs seen in Fig. 1b. In separate experiments, we investigated the effect of mutations around the TATA-like region. Mutations identical to two of the three designed by Avraham et al. (22) in the murine promotor were used. The third mutation could not be done because of the sequence difference between the two species. Mut1 (A-C) had no effect, in contrast to the 75% inhibition of activity seen by Avraham et al. (22) when the murine promotor was introduced into a rat cell line. Mut2, which introduced the sequence TATA into the gene, caused a 35% increase in activity, in contrast to the 92% reduction seen by Avraham et al. (22).

Electrophoretic Mobility Shift Assays

Analysis of the ets Site—We have performed EMSA analysis of binding to the −80 CAGGAA region. A probe containing the ets regulatory site of the rat GP IIb gene (from M. Poncz), which has no other homology to the serglycin sequence, inhibits binding to the serglycin ets probe in HEL cell nuclear extracts. An oligonucleotide in which the CAGGAA sequence was mutated to CAGCAA, the same mutation used in the mutagenesis experiments described above, did not compete with the native sequence (Fig. 2a, right lane), whereas the native sequence completely self-competed with the labeled probe (not shown). Neither a peptide representing the DNA-binding carboxyl-terminal domain nor an anti-ets-1ets-2 antibody (Santa Cruz Biotechnology, Inc.) competed with the nuclear extract for binding with the probe (not shown). CHRF nuclear extracts contained a protein that bound similarly to that of HEL cells, but HL-60 nuclear extracts bound a more quickly migrating complex (Fig. 2b) that appeared in a position similar to published reports of migration of PU.1 (53), but the usual binding site for PU.1 is GAGGAA.

Analysis of the CRE Site—Nuclear proteins of the same mobility from HEL, CHRF, and HL-60 cells bound to both the CRE oligonucleotide from the serglycin promotor and to the consensus CREB sequence (Fig. 3a). Fig. 3b shows the cross-competition between the consensus CREB oligonucleotide (54) and the serglycin putative CRE site oligonucleotide in HEL cells; identical results were obtained for the other cell lines. Thus these cell lines all appear to bind a species of CREB protein.

UV Cross-linking of Proteins to the ets/CRE Oligonucleotide—UV cross-linking of CHRF nuclear proteins to an oligonucleotide that contained both the ets and CRE sites showed cross-linking of proteins of the same size that had bound to oligonucleotides containing either site alone (Fig. 4). Two proteins bound to the ets oligonucleotide; the lower molecular mass protein, which was also the fainter band, is the major protein bound in the presence of the CRE site. The bound protein is smaller than ets-1 or ets-2 (53–56 kDa) (55), because the migration position represents the protein plus the double-stranded oligonucleotide. This protein might be, for example, erg1 or elf-1 (56, 57). There appeared to be two bands bound to the CRE oligonucleotide, and both appeared also to bind to the ets/CRE oligonucleotide. HEL extracts behaved similarly, and HL60 extracts bound only the CRE protein to the CRE or ets/CRE oligonucleotides, and no proteins were cross-linked to the ets oligonucleotide (not shown).

Binding of Nuclear Proteins to GATA and E-box Sites—We performed a number of EMSAs with oligonucleotides containing the reverse GATA site and several of the E-box sites. Specific binding of proteins to the GATA oligonucleotide was observed in HEL and CHRF but not HL60 cells. All three cell lines had nuclear proteins that bound specifically to several of the E-box oligonucleotides. However, because the transfection

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Fig. 3. EMSA for binding to the CRE oligonucleotide. a, nuclear extracts (5 μg of protein) from the three cell lines were incubated with the 32P-labeled serglycin CRE oligonucleotide (left panel) or the 32P-labeled consensus CRE oligonucleotide (right panel). The lanes marked HEL represent 5 and 10 μg of nuclear extract, b, competition between the CRE and CREB oligonucleotides. 32P-Labeled CRE (left panel) and 32P-labeled CREB (right panel). The data shown are for HEL cells; the results from all the cell lines were identical.

Fig. 4. Cross-linking of proteins to the ets, CRE, and ets/CRE oligonucleotides. The oligonucleotide is described in the text. After exposure to UV light, the samples were electrophoresed on SDS-polyacrylamide gel electrophoresis, and the protein/oligonucleotide cross-linked complexes were detected by autoradiography.

data show that these sequences are probably not key regulatory elements, the EMSA data will not be presented here.

Effects of PMA and dbcAMP on Serglycin Expression

Effect of PMA and dbcAMP on Cell Proliferation in HEL, CHRF, and HL-60 Cells—All three cell lines exhibited a lag phase of about 24 h followed by a generation time of about 24 h for HEL and CHRF and 18 h for HL-60. Treatment of all three cell lines with PMA resulted in adhesion of cells to the culture dish and no further increase in cell number. Thus after 2 days, the control cultures had about twice as many cells as the PMA treated cultures. However, HEL and CHRF cells were harvested after a 96-h incubation. 5 μg of RNA were applied to each lane. b, effect of PMA and dbcAMP on serglycin mRNA expression in HEL, CHRF, and HL-60 cells. 5 μg of RNA from HEL and CHRF and 2 μg from HL-60 cells were applied to the Northern gel.

sites, did not alter mRNA expression in any of our cell lines (not shown), in contrast to a recent report that dexamethasone increased serglycin expression in murine mast cells (58).

Effects of PMA and dbcAMP on Promoter Utilization in HEL, CHRF, and HL-60 Cells—In these experiments, cells from a single electroporation cuvette were aliquoted so that the control and the treated samples were derived from a single pool of transfected cells, eliminating the concern of differences in transfection efficiency between samples that would be compared with each other. In 15 experiments with the HEL cells, expression of all deletion constructs from −89 to −1123 and the constructs bearing mutations of the −40ets, +282CATCG, and −357GATA sites was increased 2.5 ± 0.35-fold (n = 15) in the presence of PMA. The exception was the CRE mutation, which did not give increased hGH expression in response to PMA. The electroporation process appeared to abrogate the PMA effect on serglycin synthesis in CHRF cells, because endogenous serglycin mRNA expression, expression from the serglycin promoter constructs, and proteoglycan synthesis from [35S]sulfate and overall proteoglycan size were all unchanged when the electroporated cells were cultured with PMA. In HL-60 cells, expression from the promoter constructs was virtually eliminated by PMA, with or without the CRE mutation, so the repressive effect of PMA was independent of the CRE half-site. Expression of the reporter gene from pCMVhGH in HL-60 cells, on the other hand, was increased 15–20-fold by PMA relative to an aliquot of untreated cells from the same electroporation cuvette, showing that our protocol permitted transfection of the cells and that the inhibitory effect of PMA on the serglycin promoter was specific and was not due to failure to introduce the serglycin promoter vector into the cells.

In other experiments, the effect of dbcAMP was compared with that of PMA in cells transfected with the −543/+42, the TATA region mutations, the −284/+42, and the −284/+42 ets-mut and CRE-mut. Aliquots of cells from a single electroporation cuvette were used for control and PMA and dbcAMP treatments to avoid variations in transfection efficiency among samples that were being compared with one another directly. The degree of enhancement or inhibition of reporter activity with both agents was consistent with their effects on endogenous serglycin mRNA expression, which were shown on the Northern blot in Fig. 5b. In HEL cells, the expression from all
vectors but the CRE mutants was increased ~2.5-fold by PMA and 4.4 ± 0.6-fold by dbcAMP, and activity of the CRE-muts was increased 2-fold by dbcAMP, as determined by the total hGH produced per well. Thus in HEL cells, the response of the promoter constructs to PMA and about half of the response to dbcAMP are mediated at least in part by the ~70 CRE half-site. In HL-60 cells, in contrast to the complete inhibition by PMA, dbcAMP caused a 4-fold increase in hGH expression from the ~284/+42 construct, but there was no expression with the CRE mutation construct either under unstimulated conditions or in the presence of PMA or dbcAMP. Thus the PMA effect was independent of the CRE because the activities of nonmutated and mutated constructs were all reduced, but the dbcAMP effect in HL60 cells may be mediated entirely through the CRE. In these experiments, the internal standard, pCMVSEAP (the active element of the cytomegalovirus promoter includes a CRE) was tested in HEL cells. Activation by dbcAMP was 73-fold, and activation by PMA was 26-fold; thus the effect on the cytomegalovirus promoter was much greater than the effect on the serglycin promoter, but the relative stimulations by dbcAMP and PMA were inverse to the stimulation of the serglycin promoter constructs by these agents. PMA also greatly stimulated expression of hGH from pCMVhGH (not shown).

Effect of PMA and dbcAMP on Binding of Nuclear Proteins to the ets and CRE Oligonucleotides—EMSA showed that the binding of nuclear proteins to the CRE site in all three cell lines was greater for cells treated with PMA (Fig. 6), but binding of proteins from PMA-treated HEL and CHRF cells to the ets site was reduced. PMA did not change the binding of proteins to the ets site in HL60 cells.

Effect of dbcAMP on the flt-1 Promoter in HEL Cells—To compare the activity of the flt-1 promoter with that of the serglycin promoter, HEL cells were transfected with the flt-1 promoter construct, which contained the CRE and ets sites, and the cells were subjected to treatment with PMA or dbcAMP. A 10.3 ± 1.61-fold increase in activity with dbcAMP and a 4.21 ± 1.12-fold increase with PMA (n = 6) were observed. This finding was in contrast to the finding that dbcAMP did not increase the activity of the flt-1 promoter in bovine aortic endothelial cells (59, 60).

Discussion

The expression of serglycin is up-regulated during normal megakaryocyte maturation (2, 61), in normal leukocytes during the period of granule formation (3), upon physiologic stimulation of lymphocytes (5) or stimulation of murine T-lymphocytic EL4.E1 by PMA (6), and in response to PMA in hematopoietic tumor cell lines with megakaryocytic characteristics, such as HEL (8, 45), CHRF 288-11 (9), and K562 (62). This has been shown by in situ hybridization in human bone marrow cells (3) and by radiosulfate incorporation and/or mRNA expression (2, 4, 8, 9, 45, 61, 63) in megakaryocytes and the megakaryocytic cell lines. In contrast, the myeloid cell lines, such as HL-60, down-regulate serglycin mRNA expression and protein synthesis in response to PMA (62, 64, 65). These findings are supported by nuclear run-off experiments that have shown that PMA induces increased transcription of serglycin mRNA in K562 cells but, in contrast, reduces transcription in HL-60 (62). Thus transcriptional regulation appears to be a major factor governing expression of the serglycin gene. There are no phorbol response elements in the known 5′-flanking sequences of either the human or the murine serglycin gene, and thus the effect of PMA may not be mediated by a direct action of the AP-1 proteins on this gene. We chose to examine cell lines representative of megakaryocytic and myeloid cells to explore regulation of a gene common to these cells that appears, at least in response to PMA, to have elements that are susceptible to cell-specific regulation.

This study has shown by use of deletion constructs and site-directed mutagenesis that two elements in the 5′-flanking region of the human serglycin gene, (-80)ets and (-70)CRE sites, are critical for gene expression in the cell lines that we have studied. The human serglycin promoter is configured similarly to that of known megakaryocyte/erythroid-specific genes in terms of the localization of the ets and GATA sites that are critical for these genes (28, 29, 31–33, 35–38, 44, 66) but is activated differently from these genes when expressed in the same HEL megakaryocytic cell line that has been used for most of the megakaryocytic gene expression studies. Because the GATA site of the human serglycin gene is not conserved in the murine gene, it is not surprising that this site is not a critical regulator of expression of this gene. Avraham et al. (22) had shown previously that truncation of the ets site of the murine serglycin promoter resulted in loss of activity, and Stevens et al. (67) had suggested previously that this region, which also includes a glucocorticoid response element just 5′ to the CRE, was likely to be the major regulatory region of both the human and the murine serglycin genes. Some hematopoietic cell lineage-specific regulation may occur, because serglycin gene expression increases in response to dexamethasone in murine mast cells (58); but in the murine T-lymphocytic cell line...
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The importance of the *ets* site of the murine serglycin promoter (22) was suggested initially by loss of activity of the construct in which the *ets* site was truncated in the study, but this site was not characterized. Our findings of the importance of the *ets* site in the human serglycin gene are of interest in light of the recent report that serglycin was identified as a target for *ets*-1 activation by differential display and that *ets*-1-transfected NIH 3T3 cells can activate the normally quiescent serglycin gene (71). It is likely that endogenous NIH-3T3 CREB proteins interact with the transfected *ets*-1. However, we have ruled out *ets*-1/*ets*-2 as the regulatory *ets* family proteins for the serglycin gene in our cell lines by our EMSAs with anti-*ets* antibodies. Thus far, the *ets* proteins thought to be involved in megakaryocyte gene expression are PU.1 (29) and *flt*-1 (38). PU.1 has greater mobility on EMSAs than the HEL or CHRF nuclear proteins, which bind to the *ets* oligonucleotide in our EMSAs, and has consistently been reported to bind to the sequence GAGGAA rather than CAGGAA, and *flt*-1 is larger than would be indicated by our cross-linking data.

Only two examples of regulation by closely apposed CRE and *ets* sites have been reported previously, for *flt*-1 in bovine aortic endothelial cells (59, 60) and for the transferrin receptor in differentiating murine erythroleukemia cells (72). These studies have not shown a role for cAMP. The spatial relationships of the two sites are reversed in the *flt*-1 promoter relative to their positions in the serglycin promoter; the CRE site is 5′ to the *ets* site in the *flt*-1 gene. The *flt*-1 CRE is a full canonical palindromic site TGACGTCA (59, 60), but the serglycin CRE is a half-site TGACGT. The *flt*-1 gene is also expressed in megakaryocytes (73), but its regulatory elements have not been studied in megakaryocytes. It is of interest to compare the response of the *flt*-1 promoter to dbcAMP and forskolin in bovine aortic endothelial cells (59, 60) to our data; in contrast to our finding that the expression of the *flt*-1 promoter construct in the hematopoietic cells was increased 10-fold by dbcAMP, this agent did not affect the activity of the *flt*-1 promoter in bovine aortic endothelial cells. The reason for this is not known, but the structure of the DNA may play a role. It has been shown that the TGA sequences of the CRE cause an inherent bend in DNA (74). The different responses to cAMP could be due to factors such as the presence of a full CRE in the *flt*-1 gene versus the half-site in serglycin, the reversed orientation of the sites, the changes in relative expression of proteins that bind to these sites (as we showed for PMA), or the utilization of different *ets* proteins in the complex. These factors could create different interactions between CREB or *ets* proteins with CREB-binding protein, the protein that binds to the cAMP response element-binding protein and interacts with the transcription complex. The studies that have shown interactions between closely apposed *ets* and CRE sites have not shown dependence of these promoters on elevation of cAMP levels (72, 75–77). The transferrin promoter is activated by Me2SO or N,N′-hexamethylene-bis-acetamide; N,N′-hexamethylene-bis-acetamide alters expression of protein kinases A and C in MEL cells (78), but Me2SO does not increase cAMP in MEL cells (79).

Interestingly, the EMSA data of Lok and Ponka (72) suggest that the amount of protein bound to the *ets* site relative to the CRE site decreases in N,N′-hexamethylene-bis-acetamide- or Me2SO-treated cells. This is similar to our data with PMA-stimulated HEL and CHRF cells. *ets* sites have been shown to interact with sites for other transcription factors, e.g., an *ets* site cooperates with an *Sp1* site in the megakaryocyte GP IIb promoter (29), and *ets* sites have been shown to cooperate with API sites in a number of other genes (80). CREs frequently interact with AP-1 sites, and a recent study demonstrated an interaction between a CRE site and a basic helix-loop-helix binding site in the neurospecific *vlf* promoter (81).
response to both PMA and dbcAMP and have found that the degree of change of endogenous mRNA expression in response to PMA and dbcAMP parallels the changes we have seen in the expression of the promoter constructs with these agents. We have thus provided a physiologic correlation for our findings. Previous studies with other systems have shown that PMA alone can raise cAMP levels (81, 82), and many studies have shown that PMA can act in synergy with other cAMP-elevating agents. Previous studies have reported that the effect of PMA is exerted through the CRE in the dopamine β hydroxylase (83) and DNA polymerase B (84) promoters, but there are no ets sites in these promoters. We have found that there were significant differences in the binding of nuclear proteins from the megakaryocytic cells compared with HL-60 cells to oligonucleotides representing the putative regulatory sites (Fig. 3) and the reverse GATA site (not shown). We hypothesize that the interaction between specific CRE and ets proteins, and possibly other proteins, in the megakaryocytic versus the promyelocytic cells may be involved in the disparate effects of PMA on this promoter. This could result from the changes in the relative amounts of the ets and CRE site-binding proteins, which we have detected by EMSA. This may also result from bending of the DNA in the presence of the various ets family proteins (85) or the CRE site (74) or to interaction with cell-specific factors in the initiation complex. ets-1 was shown to interact with CRE-binding protein (83), the protein that classically interacts with the CRE.

It is of interest to compare the regulatory sites of the serglycin promoter to those of other proteoglycans. Chick cartilage aggrecan is regulated by Sp1, AP-2, and NF-1-related sites (86), and rat aggrecan is regulated post-transcriptionally by cAMP (87). The mouse aggrecan gene has a high GC content and SP-1 and glucocorticoid response element sites (88). The perlecain promoter has a transforming growth factor-β-responsive element that bound to unidentified transforming growth factor-β-inducible nuclear proteins with high affinity for NF1 members of transcription factors (89). Perlecain is down-regulated by cAMP (90). The syndecan-1 promoter is activated by the Wilms’ tumor WT-1 protein (91). The biglycan gene has binding sites for Sp1, AP-1, and AP-2 factors (92). It is up-regulated by cAMP (93), but no CRE is present in the promoter, and the cAMP effect is mediated via a Sp1-like proteins (94). The DSPG3 proteoglycan may be regulated at Sox-1 sites (95). Likewise, Sp1 sites appear to be critical for expression of the mouse ryudocan gene (96) and possibly for the rat glypican-1 gene (97). The decorin gene has a purine/pyrimidine segment that is sensitive to S1 nuclease and has potential binding sites for AP-1, AP-2, and NF-kB (98). Versican has a CRE half-site near the TATA box region (47), but the enhancer element does not include that region, and there are no reports in the literature concerning the effects of cAMP or analogs on versican synthesis. Thus the regulatory elements that we have identified in the serglycin promoter are not found in common with any of the known proteoglycan genes.

Our study is the first to show DNase I hypersensitive sites in a proteoglycan gene. The HEL and CHRF cells exhibited the same pattern of DNase I hypersensitivity, but the site was stronger in CHRF cells. The HL60 had the same two sites, which appeared to be stronger than in the HEL and CHRF cells, but also a unique DHSS was found within the first intron. The unique intronic DHSS may explain the differences in the levels of endogenous expression among these three cell lines but cannot explain the differences in the effects of PMA on expression of the 5’-flanking region promoter sequences between the megakaryocytic and HL60 cells. The DHSS site around –276 was about 200 bp upstream of the ets and CRE sites. No specific elements responsible for the DNase I hypersensitivity have been defined in this region. Potential transcriptional regulation sites within 100 bp of the +825 site in the first intron include PEA-1 and AP-1 sites. The +2027 region, the DHSS region unique to HL60 cells, contains multiple potential E-box sites.

The first evidence that transcription of the serglycin gene is regulated in a complex manner in different cell types was the finding that the steady-state levels of the serglycin transcripts in fibroblasts transfected with serglycin cDNA were considerably less than in mast cells (24). Information on the regulation of serglycin expression will be of interest in understanding the function of this unique proteoglycan that is expressed widely in hematopoietic cells, in endothelial cells, and in uterine decidual cells because the level of expression in response to specific stimuli may have profound effects on modulating assembly of secretory granules or vesicles in these cells and the activity of the biologically active serglycin-binding proteins subsequent to their release. A regulatory mechanism that warrants further investigation is methylation of the DNA. Methylation patterns differ between HL-60 and non-serglycin-expressing Molt 7 T-lymphoblasts (7). Endogenous methylation of the CG pair that is part of the CRE in the serglycin gene may affect the function of the CRE. Because bacterial DNA is not methylated properly and synthetic DNA is not methylated at all, the in vitro transfection and EMSA studies would not take this possibility into account.

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