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CD43 gene expression is mediated by a nuclear factor which binds pyrimidine-rich single-stranded DNA

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ABSTRACT

CD43 is a leukocyte-specific surface molecule which plays an important role both in adhesion and signal transduction. We have identified a site spanning nucleotides +18 to +39 within the human CD43 gene promoter which *in vitro* is hypersensitive to cleavage by nuclease S1. Repeats of this region are sufficient to activate expression of a heterologous promoter in CD43-positive cell lines. Two nuclear factors, PyRo1 and PyRo2, interact with the hypersensitive site. PyRo1 is a single-stranded DNA-binding protein which binds the pyrimidine-rich sense strand. Mutation analysis demonstrates that the motif TCCCCT is critical for PyRo1 interaction. Replacement of this motif with the sequence CATATA abolishes PyRo1 binding and reduces expression of the CD43 promoter by 35% in Jurkat T lymphocytic cells and by 52% in the pre-erythroid/pre-megakaryocytic cell line K562. However, this same replacement failed to affect expression in U937 monocytic cells or in CEM T lymphocytic cells. PyRo1, therefore, exhibits cell-specific differences in its functional activity. Further analysis demonstrated that PyRo1 not only interacts with the CD43 gene promoter but also motifs present within the promoters of the CD11a, CD11b, CD11c and CD11d genes. These genes encode the α subunits of the β 2 integrin family of leukocyte adhesion receptors. Deletion of the PyRo1 binding site within the CD11c gene reduced promoter activity in T lymphocytic cells by 47%. However, consistent with our analysis of the CD43 gene, the effect of this same deletion within U937 monocytic cells was less severe. That PyRo1 binds preferentially to single-stranded DNA and sequences within the CD43 and CD11 gene promoters suggests that expression of these genes is influenced by DNA secondary structure.

INTRODUCTION

CD43 is normally expressed exclusively on the surface of leukocytes and platelets (1). The mature molecule is composed

of 381 amino acids divided between a 235 residue extracellular region, a 23 residue transmembrane region and a 123 amino acid C-terminal intracellular region (2,3). The extracellular region contains approximately 84 sialylated O-linked carbohydrate units and appears by rotary shadowing to be a large rod-like structure extending 45 nm from the cell surface (4). Comparison of the rat, mouse and human sequences indicates that the intracellular domain has been highly conserved during evolution, suggesting a critical function (2,3,5,6).

Recent evidence has indicated that CD43 serves multiple functions. As a large, abundant and highly charged molecule CD43 was originally proposed to be a barrier molecule which blocks cell–cell interactions (7). Subsequently, this proposition has been supported by two sets of experiments. In the first, introduction of CD43 into CD43-negative cells was shown to reduce CD54-mediated adhesion (8). In the second, targeted disruption of the CD43 gene in T lymphocytes was shown to enhance homotypic adhesion and binding to fibronectin and HIV-I gp120 (9). Based on these studies it has been suggested that one of the physiological roles of CD43 may be the maintenance of resting blood cells in the circulation by the prevention of adhesion. Indeed, such a survival function for CD43 has been demonstrated directly *in vivo* in transgenic mice (10). During cellular activation, the surface expression of CD43 is down-regulated by proteolytic cleavage and/or its glycosylation pattern is altered allowing cell–cell interaction (11–20). Under these circumstances, the CD43 which remains on the cell surface appears now to act as a positive regulator of cellular adhesion. This function is indicated by the finding that antibodies to CD43 activate monocytes, B lymphocytes, dendritic, mast and natural killer cells (21–27). CD43 has also been shown to activate T lymphocytes by binding MHC class I molecules and to be the cell surface component of T lymphocyte activation pathways which are independent both of the T lymphocyte receptor/CD3 complex and CD28 (28–31). The intracellular domain of CD43 likely mediates such activation through its phosphorylation by protein kinase C and its physical interaction with Fyn and Lck kinases (32–34). In addition to these positive effects on inter-leukocyte adhesion, CD43 binds galectin-1 and CD54 and, therefore, may also mediate the adhesion of leukocytes to endothelial and epithelial cells (35–37).

To determine the molecular mechanisms which control CD43 expression at the transcriptional level we and others have embarked on the characterization of the gene from which it is produced (38–42). Here we report that the activity of the

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human CD43 gene promoter is dependent upon a region which *in vitro* is hypersensitive to cleavage by nuclease S1. Two nuclear factors, PyRo1 and PyRo2, bind within this region. PyRo1 also interacts with all four genes encoding the α subunits of the β 2 integrin family, suggesting a common mechanism of regulation.

MATERIALS AND METHODS

Plasmid construction

The expression constructs p43Wt, p43 Δ P, p43 μ P, p11Wt and p11 Δ P were generated by cloning fragments of the CD43 and CD11c genes upstream of the promoterless luciferase gene present in the plasmid pATLuc (43). Specifically, the construct p43Wt was produced by cloning into the 'filled-in' *Hind*III site of pATLuc a PCR product spanning nucleotides -2 to +99 of the CD43 gene. The plasmid p43 Δ P was then generated by employing a two-step cloning strategy. First, a plasmid containing nucleotides -990 to +99 of the CD43 gene was treated with nuclease S1 resulting in a linearized DNA fragment which was subsequently re-ligated to yield the plasmid p43S1. Next, the promoter region of the CD43 gene spanning nucleotides -2 to +99 was amplified from p43S1 and cloned into the 'filled-in' *Hind*III site of pATLuc to produce the plasmid p43 Δ P. DNA sequencing of this plasmid demonstrated that it contained a deletion of nucleotides +18 to +39. The construct p43 μ P was produced by cloning into the 'filled-in' *Hind*III site of pATLuc annealed complementary oligonucleotides representing nucleotides -2 to +99 of the CD43 gene containing the mutant sequence CATATA between nucleotides +32 and +37. The constructs p11Wt and p11 Δ P were produced by cloning into the 'filled-in' *Hind*III site of pATLuc PCR products representing, respectively, nucleotides -128 to +36 and -117 to +36 of the CD11c gene. The plasmid pGL3-HYP was generated by cloning four direct repeats of the +18/+39 hypersensitive region into the *Xho*I site of pGL3-Promoter Vector (Promega Corp., Madison, WI). This site is located immediately upstream of the SV40 promoter which drives basal expression of a luciferase reporter gene. The correct orientation and nucleotide sequence of all constructs was verified by DNA sequencing.

Cell culture

The pro-megakaryocytic cell line MEG-01 (44) was provided by Dr W. S. May (Johns Hopkins Oncology Center, Baltimore, MD) with permission from Dr H. Saito (Nagoya University School of Medicine, Nagoya, Japan). MEG-01 cells were cultured in RPMI 1640 supplemented with 20% fetal calf serum, aqueous penicillin G (100 U/ml) and streptomycin (50 mg/ml). Additional cell lines were obtained from the American Type Culture Collection and grown according to their specifications. Phorbol 12-myristate 13-acetate (PMA) was obtained from the Sigma Chemical Co. (St Louis, MO) and used at a concentration of 100 ng/ml where indicated.

Transfection

Cells were transfected with 23 μ g of a given luciferase reporter plasmid and 2 μ g of the plasmid pRSV- β containing the *lacZ* gene as described by Farokhzad *et al.* (45). After transfection, cells were grown for 16 h in 30 ml of complete medium

containing or lacking PMA and processed for the assay of luciferase and β -galactosidase activity (46).

Detection of the nuclease S1 hypersensitive site

Ten micrograms of a supercoiled plasmid, containing the CD43 gene spanning nucleotides -990 to +99 relative to the 5' major transcription initiation site, were incubated at 42°C with no nuclease S1 or 40 U of the enzyme. The samples were then digested with *Eco*RV or *Sph*I and subjected to agarose gel electrophoresis. Comparison of the fragmentation patterns of nuclease S1-treated samples with those of equivalent untreated samples identified fragments resulting from the dual digestion of a restriction endonuclease and nuclease S1. In order to precisely map the nuclease S1 hypersensitive site, the CD43 gene clone was treated with nuclease S1 as described above, the cleavage sites 'polished' with T4 DNA polymerase in the presence of deoxynucleotides and then re-ligated. DNA sequencing of the resulting plasmids indicated that the nuclease S1 hypersensitive region extends from nucleotides +18 to +39.

Preparation of nuclear extracts

Approximately 250 million cells were collected by centrifugation, washed three times in ice-cold phosphate-buffered saline (PBS) and resuspended in 4 ml of ice-cold buffer 1 (10 mM NaCl, 0.4 M sucrose, 10 mM Tris-HCl, pH 7.8, 0.2 mM EDTA, 0.1 mM EGTA, 0.5% NP-40, 0.5 mM PMSF, 1 mg/ml pepstatin, 1 mg/ml leupeptin, 50 mg/ml antipain and 1 mg/ml aprotinin). The resuspended cells were then incubated on ice for 30 min and layered over 4 ml of ice-cold buffer 2 (buffer 1 containing 1.5 M sucrose and 0.5 mM DTT but no NP-40). Nuclei were collected by centrifugation, washed with 4 ml of ice-cold buffer 3 (buffer 1 containing 0.5 mM DTT but no NP-40) and resuspended to a concentration of 1×10^8 cell equivalents/300 μ l in ice-cold buffer 4 (20 mM Tris-HCl, pH 7.8, 300 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 25% glycerol and the protease inhibitor cocktail listed above). Resuspended cells were then incubated at 4°C for 60 min and dialyzed overnight at 4°C against buffer 4. The dialyzed nuclear extract was clarified by centrifugation, frozen in liquid nitrogen and stored at -80°C. The concentration of protein present in the nuclear extracts was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides were radiolabeled at their 5'-ends using T4 polynucleotide kinase and purified through G-25 Sephadex columns. In order to generate double-stranded DNA, equimolar amounts of complementary oligonucleotides were combined. These oligonucleotides were then annealed by the addition of 5 M NaCl to a final concentration of 100 mM, heating to 90°C and slow overnight cooling to 4°C. DNA-protein binding reactions were carried out in a 20 μ l volume. First, nuclear extracts were incubated with or without a molar excess of unlabeled competitor probe at 4°C for 15 min in 70 mM KCl, 5 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 10% glycerol and 2.4 μ g poly(dI:dC)-poly(dI:dC). Radiolabeled probe was then added and the incubation continued for 30 min. The DNA-protein complexes were resolved by electrophoresis through 7% native polyacrylamide gels and visualized

by autoradiography. The double- and single-stranded oligonucleotides used in these analyses were:

- CD11a GAGA: 5'-GCACACCTCCCTCCCCGCCTG-3' (43)
3'-CGTGTGGAGGGAGGGGCGGAC-5'
- CD11a LYM: 5'-TTTTGGATGATGTGAAAATGCAAG-3' (43)
3'-AAAACCTACTACACTTTTACGTTTC-5'
- CD11a PyRo SS: 5'-CAAATCCCACGGGCTCCTGACG-3' (this paper)
- CD11b PyRo ASS: 5'-GCCTGCCACCTTCTCCCCAGCTT-3' (this paper)
- CD11c Ets: 5'-CACTTGCTTCTCAGTACC-3' (this paper)
3'-GTGAACGAAGGAGTCATGG-5'
- CD11c PyRo: 5'-TGGGGGTGGGGGCGTGTG-3' (this paper)
3'-ACCCCCACCCCGCACAC-5'
- CD11c PyRo ASS: 5'-CACACGCCCCACCCCA-3' (this paper)
- CD11c PyRo SS: 5'-TGGGGGTGGGGGCGTGTG-3' (this paper)
- CD11c Sp: 5'-CGTGTGGGAGGCCGAGCCT-3' (this paper)
3'-GCACACCTCCGGTCCGA-5'
- CD11d PyRo SS: 5'-TCCTACCCACTGTGCCCTCCTC-3' (this paper)
- CD18 Box A: 5'-CACACTTCTCCAAGGAG-3' (47)
3'-GTGGTGAAGGAGTTCCTC-5'
- CD43 PyRo: 5'-GGGCCACTTCCCTTCCCCTTG-3' (this paper)
3'-GGGTGAAGGAAAGGGGAAC-5'
- CD43 PyRo ASS: 5'-CAAGGGAAAGGAAGTGGGCC-3' (this paper)
- CD43 PyRo SS: 5'-GGGCCACTTCCCTTCCCCTTG-3' (this paper)
- CD43 Mut-01: 5'-GGGGGTGTTCCCTTCCCCTTG-3' (this paper)
- CD43 Mut-02: 5'-GGGCCACAAGGAATCCCCTTG-3' (this paper)
- CD43 Mut-03: 5'-GGGCCACTTCCCTAGGGGATG-3' (this paper)
- CD43 Mut-04: 5'-GGGGGTGAAGGAATCCCCTTG-3' (this paper)
- CD43 Mut-05: 5'-GGGCCACAAGGAAAGGGGATG-3' (this paper)
- CD43 Mut-06: 5'-GGGGGTGTTCCCTAGGGGATG-3' (this paper)
- CD43 Mut-07: 5'-GGGGGTGAAGGAAAGGGGATG-3' (this paper)
- CD43 Mut-08: 5'-GGGCCACTTCCCTTCTTTCTG-3' (this paper)
- CD43 Mut-09: 5'-GGGCCACTTCCCTCTAATCTG-3' (this paper)
- CD43 Mut-10: 5'-GGGCCACTTCCCTCATTACTG-3' (this paper)
- CD43 Mut-11: 5'-GGGCCACTTCCCTCATATATG-3' (this paper)
- Consensus AP-1: 5'-CTAGTGATGAGTCAGCCGATC-3' (Stratagene,
3'-GATCACTACTCAGTCGGCTAG-5' La Jolla, CA)
- Consensus AP-2: 5'-GATCGAAGTGCAGCCCGCGCCCGT-3' (Stratagene)
3'-CTAGCTTGACTGGCGGCGCCGGCA-5'
- Consensus AP-3: 5'-CTAGTGGGACTTTCCACAGATC-3' (Stratagene)
3'-GATCACCTGAAAGGTGTCTAG-5'
- Consensus CREB: 5'-GATTGGCTGACGTCAGAGAGCT-3' (Stratagene)
3'-CTAACCGACTGCAGTCTCTCGA-5'
- Consensus GRE: 5'-GATCAGAACACAGTGTCTCTA-3' (Stratagene)
3'-CTAGCTTGTGTCAAGAGAT-5'
- Consensus NF-1: 5'-ATTTGGCTTGAAGCCAATATG-3' (Stratagene)
3'-TAAACCGAAGTTCGGTTATAC-5'
- Consensus NF-κB: 5'-GATCGAGGGACTTCCCTAGC-3' (Stratagene)
3'-CTAGCTCCCTGAAAGGATCG-5'
- Consensus Oct-1: 5'-GATCGAATGCAATCAGTACT-3' (Stratagene)
3'-CTAGCTTACGTTAGTGATCGA-5'
- Consensus Sp1: 5'-GATCGATCGGGGCGGGCGATC-3' (Stratagene)
3'-CTAGCTAGCCCCGCCCCGCTAG-5'
- MHC PU.1: 5'-GATCCGTCCCAAGTGAAGAACCAATCAGCATTG-3' (48)
3'-CTAGGAGGGTTCCTCCTTGGTTAGTCGTAAC-5'
- NS-SS: 5'-GAGTTAGCTCACTCATTAGG-3' (this paper)
- SV40 PU.1: 5'-CCTCTGAAAGAGGAACTTGGT-3' (49)
3'-GGAGACTTCTCCTTGAACCA-5'

RESULTS

Expression of the proximal promoter of the CD43 gene in leukocytic cell lines

Previous studies have determined that the proximal promoter of the CD43 gene is sufficient to drive expression in Jurkat T lymphocytes, K562 pre-erythroid/pre-megakaryocytic cells, HeLa epithelial cells and Raji B lymphocytes (39–42). In order to determine whether the promoter also contains the *cis*-acting elements responsible for monocytic expression, the construct p43Wt was made. This contained nucleotides –2 to +99 relative to the most 5' major transcription start site of the CD43 gene fused to the 5'-end of a promoterless luciferase gene. The p43Wt construct was transfected into U937 pro-monocytic cells which were subsequently either left untreated or induced to differentiate along the monocytic pathway by treatment with PMA. Measurement of luciferase activity demonstrated that in untreated U937 cells p43Wt directs expression which is 338-fold higher than that directed by the parent plasmid. In PMA-treated cells p43Wt directs a level of expression which is 279-fold higher than its parent. Such expression in U937 cells compares favorably to the 309- and 698-fold above parental expression levels directed by p43Wt in Jurkat and K562 cells, respectively. When p43Wt was transfected into CEM T lymphocytic cells it directed a level of expression only 74-fold above that directed by its parent. This low level of promoter activity compared to other cell types is reflected in the relatively low level of CD43 protein produced by CEM cells (16). In summary, therefore, our transfection analysis indicates that the region of the CD43 gene extending from –2 to +99 is sufficient *in vitro* to direct appropriate expression in cell lines representing a range of leukocyte lineages (Fig. 1).

A region hypersensitive to nuclease S1 is present within the CD43 gene promoter

Between the two major transcription initiation sites of the CD43 gene there is an inverted repeat of the sequence CAGGGCCC which has the potential to form the stems of a cruciform (38). Within this region there is also a direct repeat of the sequence GGGCCC which might be involved in the formation of a 'slippage' structure. In addition, the sense strand between the inverted repeat is composed predominantly of pyrimidine residues and the antisense strand predominantly of purine residues. This strand asymmetry linked with the presence of mirror repeats of the sequences TTCC and TCCC could favor the formation of a triple helix (H-DNA). If cruciform, slippage or H-DNA structures were to exist within the CD43 promoter then their loop regions would be single-stranded and hypersensitive to cleavage by single strand-specific nucleases. Nuclease S1 treatment of a supercoiled plasmid containing the CD43 promoter demonstrates that *in vitro* the region between the inverted repeat does indeed exhibit such hypersensitivity (Fig. 2). Fine mapping of the hypersensitive region establishes that it spans nucleotides +18 to +39.

The nuclease S1 hypersensitive region is essential for CD43 promoter activity

Since nuclease S1 hypersensitive regions are often coincident with *cis*-acting elements critical to gene expression (50–52) we sought to determine whether this was true of the hypersensitive region within the CD43 gene promoter. As a result we generated

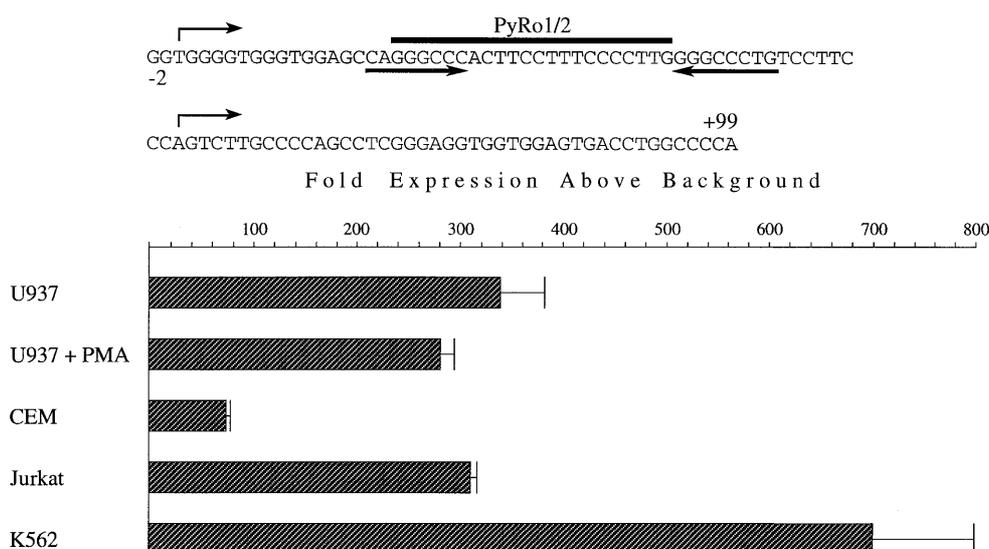


Figure 1. Activity of the proximal promoter region of the CD43 gene in hematopoietic cell lines. The nucleotide sequence of the CD43 gene promoter is depicted at the top of the figure. Bent arrows mark the major sites of transcription initiation and converging arrows the inverted repeat which might form the stems of a cruciform structure. The region which interacts with the nuclear factors PyRo1 and PyRo2 is marked above the sequence with a filled bar. Numbering is relative to the 5' major transcription initiation site (38). The construct p43Wt was generated by inserting nucleotides -2 to +99 of the CD43 gene promoter upstream of the promoterless luciferase gene present in the vector pATLuc (43). U937, CEM, Jurkat and K562 cells were transfected with p43Wt in parallel with pATLuc. U937 cells were then either left untreated or treated for 16 h with PMA to induce monocytic differentiation. The bottom of the figure depicts the level of luciferase activity directed by p43Wt above that directed by pATLuc after correction for transfection efficiency. Each histogram represents the mean \pm SD of three independent experiments.

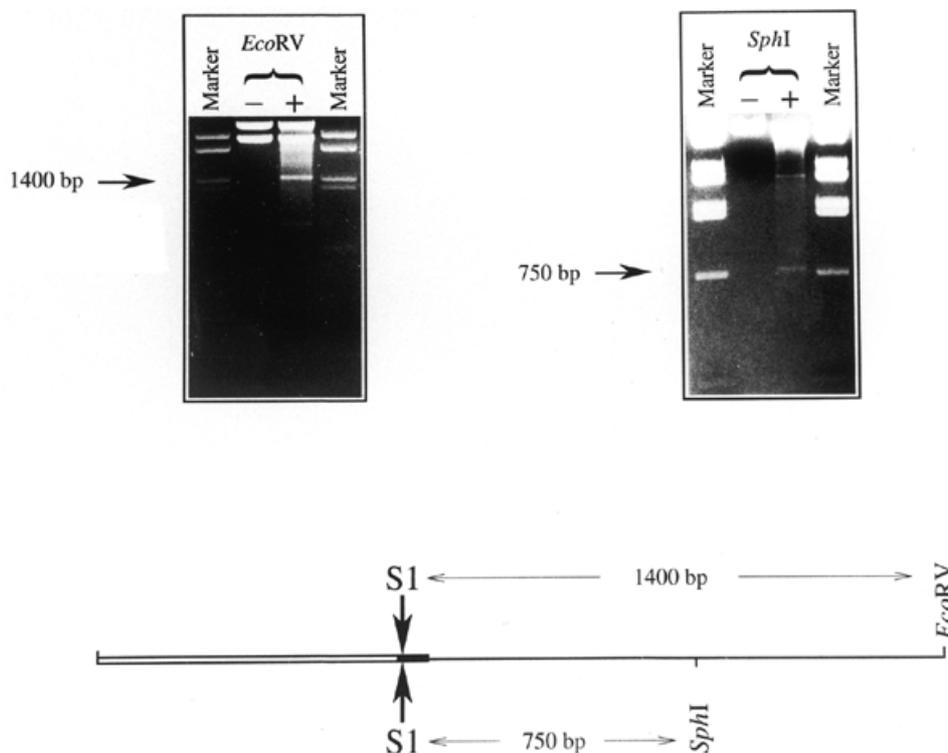


Figure 2. Detection of a site within the CD43 promoter hypersensitive to nuclease S1. A supercoiled plasmid spanning nucleotides -990 to +99 of the CD43 gene (38) was incubated with or without nuclease S1 and then digested with *EcoRV* or *SphI*. Comparison of the fragmentation patterns of nuclease S1-treated samples with those of equivalent untreated samples indicated an *EcoRV*/S1-specific fragment of 1400 bp and a *SphI*/S1 fragment of 750 bp. Mapping of the nuclease S1-specific fragments indicates the presence of a single site hypersensitive to cleavage with nuclease S1 located between the two major sites of transcription initiation. The fragmentation patterns as visualized by ethidium bromide staining are depicted at the top of the figure and a schematic representation of these patterns at the bottom. Vector sequences are indicated by horizontal lines and exon and 5'-flanking sequences of the CD43 gene are represented by filled and open boxes, respectively. Sequencing of plasmids resulting from nuclease S1 treatment followed by re-ligation indicated that the nuclease S1 hypersensitive region extends from nucleotides +18 to +39.

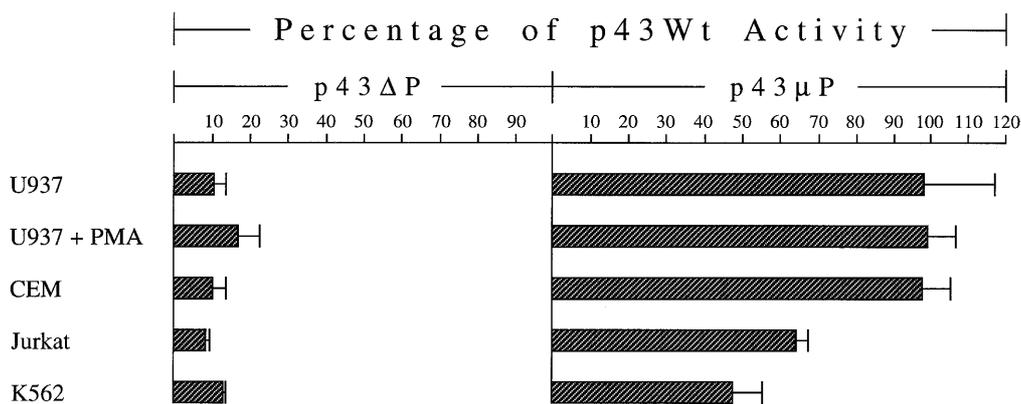


Figure 3. Contribution of the nuclease S1 hypersensitive region to the transcriptional activity of the CD43 promoter. The wild-type CD43 promoter spanning nucleotides -2 to $+99$ is present in the luciferase expression construct p43Wt, while p43ΔP represents the specific removal of the nuclease S1 hypersensitive region extending from nucleotides $+18$ to $+39$. The construct p43μP represents the specific replacement of the sequence TCCCCT, extending from nucleotides $+32$ to $+37$, with the sequence CATATA. This replacement abolishes PyRo1 binding (Fig. 7). All CD43 expression constructs were transfected in parallel with pATLuc into U937, CEM, Jurkat and K562 cells. U937 cells were then either left untreated or treated for 16 h with PMA to induce monocytic differentiation. The levels of luciferase gene activity directed by p43ΔP and p43μP above those conferred by pATLuc and after correction for transfection efficiency are expressed as histograms. The level of expression of p43Wt is assigned an arbitrary value of 100% and the expression levels conferred by p43ΔP and p43μP are displayed as proportions of this value. Each histogram represents the mean \pm SD of three independent transfection experiments.

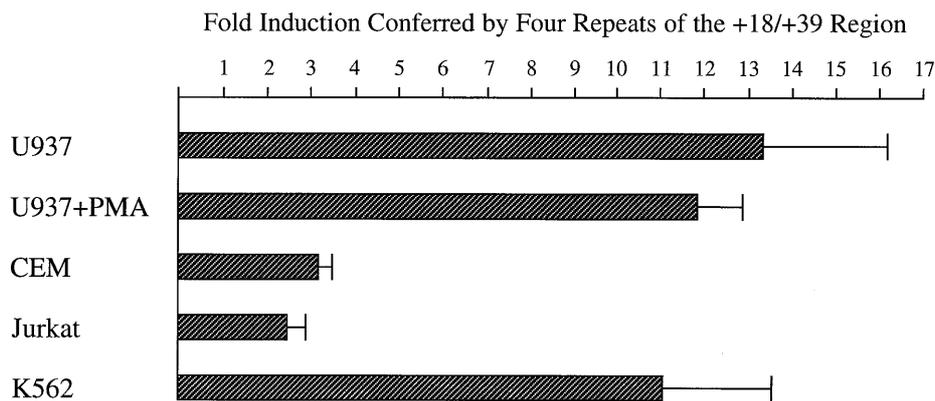


Figure 4. Induction of the transcriptional activity of a heterologous promoter by the nuclease S1 hypersensitive region. Four direct repeats of the $+18/+39$ hypersensitive region were cloned immediately upstream of the SV40 promoter present in the plasmid pGL3-Promoter Vector (Promega Corp.). The resulting construct, pGL3-HYP, was then transfected in parallel with its parent into U937, CEM, Jurkat and K562 cells. U937 cells were then either left untreated or treated for 16 h with PMA. Histograms depict the levels of luciferase activity directed by pGL3-HYP above those conferred by pGL3-Promoter Vector following a correction for variations in transfection efficiency. Each histogram represents the mean \pm SD of three independent transfection experiments.

the construct p43ΔP in which nucleotides $+18$ to $+39$, representing the hypersensitive region, are deleted from the $-2/+99$ promoter. Transfection of p43ΔP into U937 cells, either untreated or subsequently treated with PMA, or into untreated CEM, Jurkat and K562 cells demonstrated that it directed levels of luciferase activity which were on average 88% lower than those directed by p43Wt (Fig. 3, left). Consequently, nucleotides $+18$ to $+39$, which *in vitro* are hypersensitive to nuclease S1 cleavage, appear critical to CD43 gene expression.

The nuclease S1 hypersensitive region is sufficient to induce the activity of a heterologous promoter

Deletion of the nuclease S1 hypersensitive region demonstrates that its primary sequence and/or the structure it imparts are necessary for transcriptional activity of the CD43 promoter.

Next we sought to determine whether this region also contains a complement of *cis*-acting elements sufficient to activate transcription. This was achieved by generating the construct pGL3-HYP. Here four head-to-tail copies of the hypersensitive region are cloned immediately upstream of a SV40 promoter which drives basal transcription of the firefly luciferase gene. The pGL3-HYP construct was transfected into U937 cells, either left untreated or subsequently treated with PMA, as well as into CEM, Jurkat and K562 cells. After correction for transfection efficiency, the level of luciferase activity directed by pGL3-HYP in these cells was found to be 13.3-, 11.8-, 3.2-, 2.5- and 11.0-fold higher, respectively, than that directed by the parent plasmid which contains no CD43 gene sequences (Fig. 4). Consequently, the hypersensitive region of the CD43 gene promoter appears to contain elements sufficient to activate

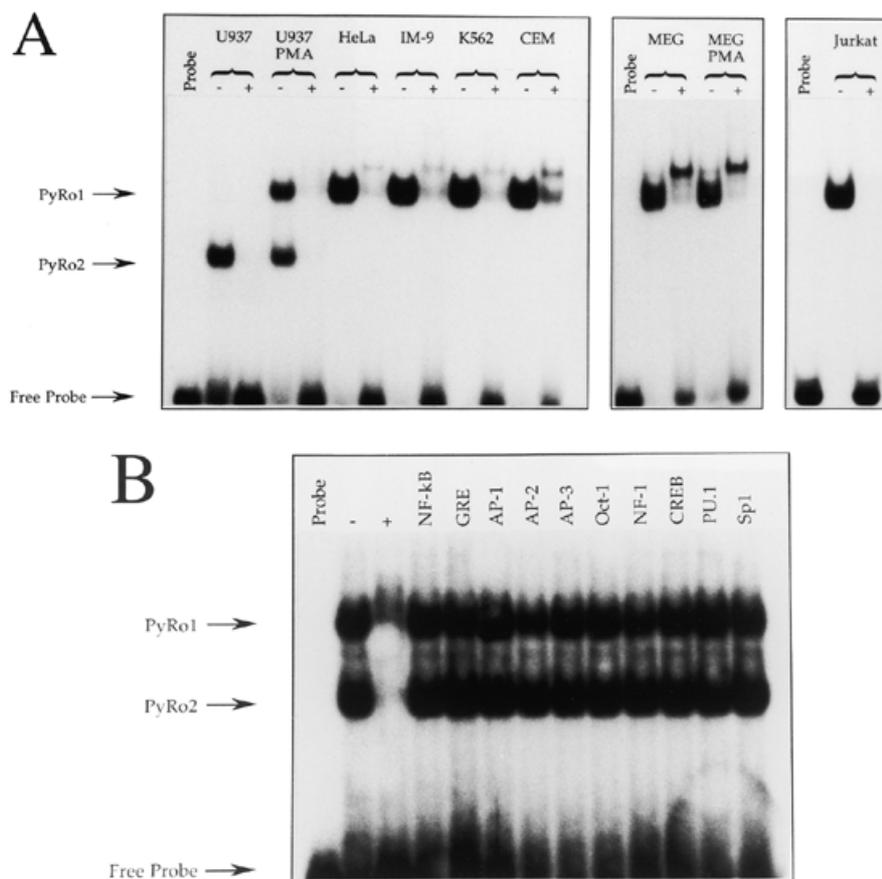


Figure 5. EMSA analysis of the nuclear proteins which interact with the nuclease S1 hypersensitive region within the CD43 gene promoter. **(A)** A radiolabeled DNA fragment, CD43 PyRo, containing the nuclease S1 hypersensitive site of the CD43 promoter, was incubated with no nuclear extract (Probe) or with nuclear extracts in the presence (+) or absence (-) of a 50-fold molar excess of its unlabeled equivalent. Nuclear extracts were prepared from U937 pro-monocytic cells, U937 cells induced to differentiate along the monocytic pathway by treatment with PMA, HeLa epithelial cells, IM-9 B lymphocytic cells, K562 pre-erythroid/pre-megakaryocytic cells, CEM T lymphocytic cells, MEG-01 megakaryocytic cells and MEG-01 cells induced with PMA to differentiate further along the megakaryocytic pathway. In addition, binding reactions were performed with a nuclear extract prepared from Jurkat T lymphocytic cells and the radiolabeled single-stranded oligonucleotide CD43 PyRo SS, which represents the sense strand of CD43 PyRo. The DNA-protein complexes PyRo1 and PyRo2 are arrowed, as is the DNA free of protein. **(B)** The specificity of the interaction of PyRo1 and PyRo2 with the CD43 promoter was assessed by attempted competition of these interactions using a 50-fold molar excess of consensus binding sites for NF- κ B, GRE, AP-1, AP-2, AP-3, Oct-1, NF-1, CREB and Sp1 (Stratagene). Competition was also performed using the double-stranded oligonucleotide MHC PU.1 (PU.1), which represents a binding site for PU.1 within the MHC class II gene I-A β (48). All competition studies were performed using radiolabeled CD43 PyRo and nuclear extracts prepared from PMA-treated U937 cells.

transcription in U937 and K562 cells, which represent the myeloid lineage. However, these same elements appear much less effective in CEM and Jurkat cells, representing the lymphoid lineage.

The +18/+39 region of the CD43 gene interacts with two nucleoprotein complexes

Since the +18/+39 region represents an important *cis*-acting element within the CD43 gene we determined by EMSA analysis whether it interacts with nuclear proteins which might mediate its function. This analysis indicated that the +18/+39 region interacts with two nuclear factors (Fig. 5). Since the +18/+39 region is rich in pyrimidine residues these factors were named PyRo1 and PyRo2, for pyrimidine recognition 1 and 2. PyRo1 is expressed in all cell types tested but in U937 promonocytic cells only after differentiation has been induced by PMA. PyRo2 is expressed exclusively in U937 cells, both uninduced

and induced with PMA. The specificity of the interaction of PyRo1 and PyRo2 with the CD43 promoter is apparent from the lack of competition of these interactions by the binding sites of a range of other nuclear proteins (Fig. 5B).

PyRo1 binds single-stranded DNA

The PyRo1/2 binding site within the CD43 promoter is hypersensitive to cleavage by nuclease S1 and, therefore, can exist as single-stranded DNA (Fig. 2). EMSA analysis was performed using either the sense or the antisense strand of the +18/+39 region of the CD43 gene in order to determine whether PyRo1 and/or PyRo2 could interact with DNA in such a conformation. This analysis demonstrated that PyRo1 interacts with the pyrimidine-rich sense strand of the +18/+39 region but not the purine-rich antisense strand (Figs 5A, right, and 6). Furthermore, when the labeled sense strand is annealed with a 250 molar excess of unlabeled antisense strand to yield a

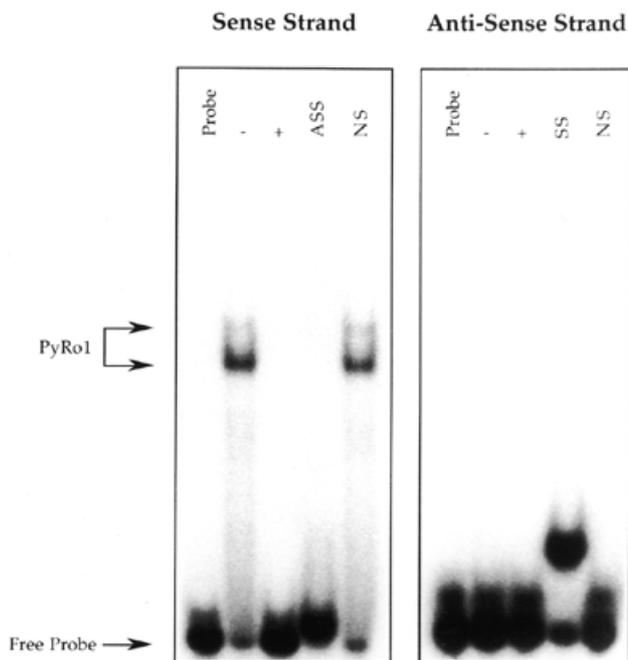


Figure 6. EMSA analysis demonstrating that PyRo1 interacts with the sense but not the antisense strand of the CD43 gene promoter. (Left) A radiolabeled single-stranded oligonucleotide, CD43 PyRo SS, representing the sense strand of the +18/+39 region of the CD43 gene was incubated with no nuclear extract (Probe) or a nuclear extract prepared from U937 cells induced for 24 h with PMA. Binding reactions were performed in the absence (-) or presence (+) of a 250-fold molar excess of unlabeled CD43 PyRo SS, in the presence of unlabeled CD43 PyRo ASS (ASS), which represents the complementary sequence of CD43 PyRo SS, or in the presence of the non-specific single-stranded oligonucleotide NS-SS (NS). The DNA-protein complexes marked with arrows have relative mobilities consistent with that of PyRo1 (Fig. 5). (Right) EMSA analysis performed exactly as described for the left panel except that CD43 PyRo ASS was employed as the radiolabeled probe and CD43 PyRo SS (SS) was used as unlabeled competitor.

labeled probe which is overwhelmingly double-stranded, PyRo1 binding is largely abolished (Fig. 6). Therefore, PyRo1 appears to interact preferentially with single-stranded DNA which is composed predominantly of pyrimidine residues. That PyRo1 binding is observed when equimolar amounts of the sense and antisense strands of the +18/+39 region are annealed is probably due to a proportion of the sense strand remaining single-stranded (Fig. 5A, left and center).

Nucleotides critical for PyRo1 binding lie at the 3'-end of the nuclease S1 hypersensitive region

Three clusters of mutations were introduced into the hypersensitive region in order to determine the nucleotides critical for PyRo1 binding (Fig. 7). The cluster of mutations at the 5'-end representing C→G and A→T transitions had no effect on the binding of PyRo1 (Mut-01). This finding is consistent with nucleotides in this area representing part of repeat sequences which within cruciform, slippage or triple helix structures would be double-stranded and unavailable for PyRo1 binding. The central cluster of mutations representing T→A and C→G transitions disrupted PyRo1 binding, indicating that nucleotides in this area are important for binding (Mut-02). However, when

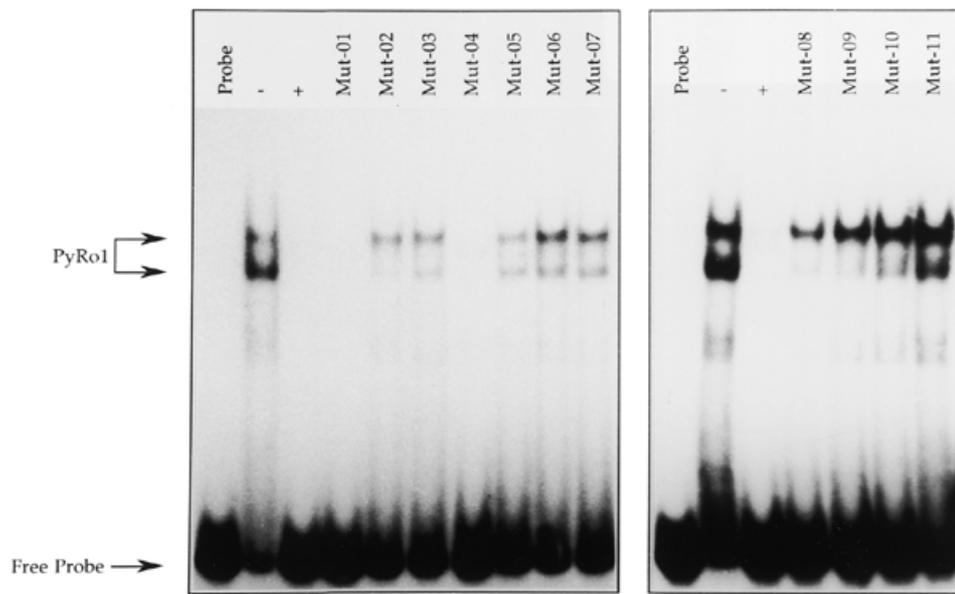
this cluster of mutations was present along with the 5'-cluster, PyRo1 binding was retained (Mut-04). Consequently, mutations in the central area can be compensated by mutations in an adjacent area. This suggests that the central area of the hypersensitive site does not directly contact PyRo1 but possibly facilitates binding by providing a specific structural context. This conclusion is supported by the finding that in isolation the central area cannot bind PyRo1 (Mut-06). Similar to the central cluster of mutations, the cluster of mutations at the 3'-end of the hypersensitive site representing T→A and C→G transitions also disrupts PyRo1 binding (Mut-03). However, unlike the nucleotides within the central area, those within the 3'-end are sufficient to bind PyRo1 in isolation (Mut-04). Therefore, the 3'-area, consisting of the hexamer TCCCCT, likely directly contacts PyRo1. Furthermore, when this motif was changed to CTTTTTC such that its pure pyrimidine character remained intact, PyRo1 binding was again lost (Mut-08). Consequently, PyRo1 appears to recognize a specific nucleotide sequence rather than a general base composition. That this is the case is supported by the observation that changing the TCCCCT sequence to CTAATC, CATTAC or CATATA all disrupt its ability to bind PyRo1 (Mut-09, Mut-10 and Mut-11). When the TCCCCT motif was replaced with the latter mutant sequence in the context of the -2/+99 promoter region, expression was reduced by 35% in Jurkat T lymphocytic cells and by 52% in the pre-erythroid/pre-megakaryocytic cell line K562. However, this same replacement failed to affect expression in U937 monocytic cells or CEM T lymphocytic cells (Fig. 3, p43μP). PyRo1, therefore, exhibits cell-specific differences in its functional activity.

PyRo1 interacts with the promoter of the CD11c gene

We have shown that the promoter region of the CD11c gene extending from -128 to +36 relative to the major site of transcription initiation is sufficient to drive a pattern of tissue-, cell- and development-specific expression *in vitro* which mimics that of the CD11c gene *in vivo* (C.S.Shelley, unpublished results). EMSA analysis of the nuclear proteins which interact with this promoter revealed one factor binding between nucleotides -128 and -110 which is constitutively expressed in all cell types tested except U937 cells, where it is only expressed after PMA treatment (Fig. 8A). This expression profile is identical to that of PyRo1. Furthermore, the binding of this factor to the CD11c promoter is effectively competed by the PyRo1 binding site within the CD43 promoter (Fig. 8B). In a complementary experiment we found that the binding of PyRo1 to the CD43 sense strand is effectively competed by the pyrimidine-rich antisense strand of the CD11c -128/-110 region but not by the purine-rich sense strand (Fig. 10). These results indicate that factors indistinguishable from PyRo1 interact with both the CD43 and CD11c gene promoters.

PyRo1 mediates CD11c promoter activity

The -128/+36 promoter of the CD11c gene is active in both PMA-treated U937 cells and PMA-treated Jurkat T lymphocytes. The PyRo1 binding site within this promoter is encompassed by nucleotides -128 to -110. In order to determine the role of PyRo1 in controlling CD11c promoter activity the luciferase reporter construct p11ΔP was generated in which nucleotides -128 to -118 were deleted from the -128/+36 promoter region. Transfection of p11ΔP into U937 and Jurkat



CD43 PyRo SS	5' -GGGCCCACTTCCTTTCCCCTTG-3'
Mut-01	5' -GGC CGGTC TTTCCTTTCCCCTTG-3'
Mut-02	5' -GGGCCCA C AAGGAA T CCCCTTG-3'
Mut-03	5' -GGGCCCACTTCCTT AGGGGA TG-3'
Mut-04	5' -GGC CGGTGAAGGA TTCCCCTTG-3'
Mut-05	5' -GGGCCCA C AAGGAA ACGGGA TG-3'
Mut-06	5' -GGC CGGTC TTTCCTT AGGGGA TG-3'
Mut-07	5' -GGC CGGTGAAGGAAACGGGA TG-3'
Mut-08	5' -GGGCCCACTTCCTT C TTTTC TG -3'
Mut-09	5' -GGGCCCACTTCCTT C TAA TCTG -3'
Mut-10	5' -GGGCCCACTTCCTT C ATT TACTG -3'
Mut-11	5' -GGGCCCACTTCCTT C ATATA TG -3'

Figure 7. Determination of nucleotides critical for PyRo1 binding. The radiolabeled single-stranded oligonucleotide CD43 PyRo SS, representing the sense strand of the +18/+39 region of the CD43 gene, was incubated with no nuclear extract (Probe) or a nuclear extract prepared from U937 cells induced for 24 h with PMA. Binding reactions were performed in the absence (-) or presence (+) of a 200-fold molar excess of unlabeled CD43 PyRo SS or in the presence of a 200-fold molar excess of one of a series of 11 unlabeled single-stranded mutant oligonucleotides (Mut-01–Mut-11). The sequences of these mutant oligonucleotides are depicted at the bottom of the figure with nucleotides different from those of the wild-type +18/+39 region boxed.

cells which were subsequently treated with PMA demonstrated that it directed levels of luciferase activity which were on average 31 and 47% lower, respectively, than those directed by the wild-type construct (Fig. 9). PyRo1, therefore, appears to contribute to expression of the CD11c promoter in both lymphocytic and monocytic cells. However, as with the CD43 gene, its contribution to monocytic expression is less than that to lymphocytic expression.

PyRo1 interacts with the CD11a, CD11b and CD11d genes

Within the CD43 gene PyRo1 binds the sense strand 5'-GGGCCCACTTCCTTTCCCCTTG-3' and within the CD11c gene it binds the antisense strand 5'-CACACGCCCCACCCC-CCA-3'. Comparison of these strands reveals that both contain

a motif consisting of the sequence CCCAC present upstream of a run of four cytosine residues. Mutation analysis of the CD43 sequence indicates that the CCCC motif is critical for PyRo1 binding *in vitro* while the CACCC motif is dispensable (Fig. 7). However, *in vivo* the CACCC motif may play an important role in PyRo1 binding, possibly by helping to produce a local single-stranded DNA conformation. On the premise that both the CACCC and CCCC sequences are involved in PyRo1 binding *in vivo* we sought to identify PyRo1 binding sites within genes other than those encoding CD11c and CD43. Specifically, since the CD11c gene is closely related to those encoding CD11a, CD11b and CD11d, these were analyzed. In every case at least one region where a CACCC motif lies close upstream of a run of cytosine residues was identified. Single-stranded

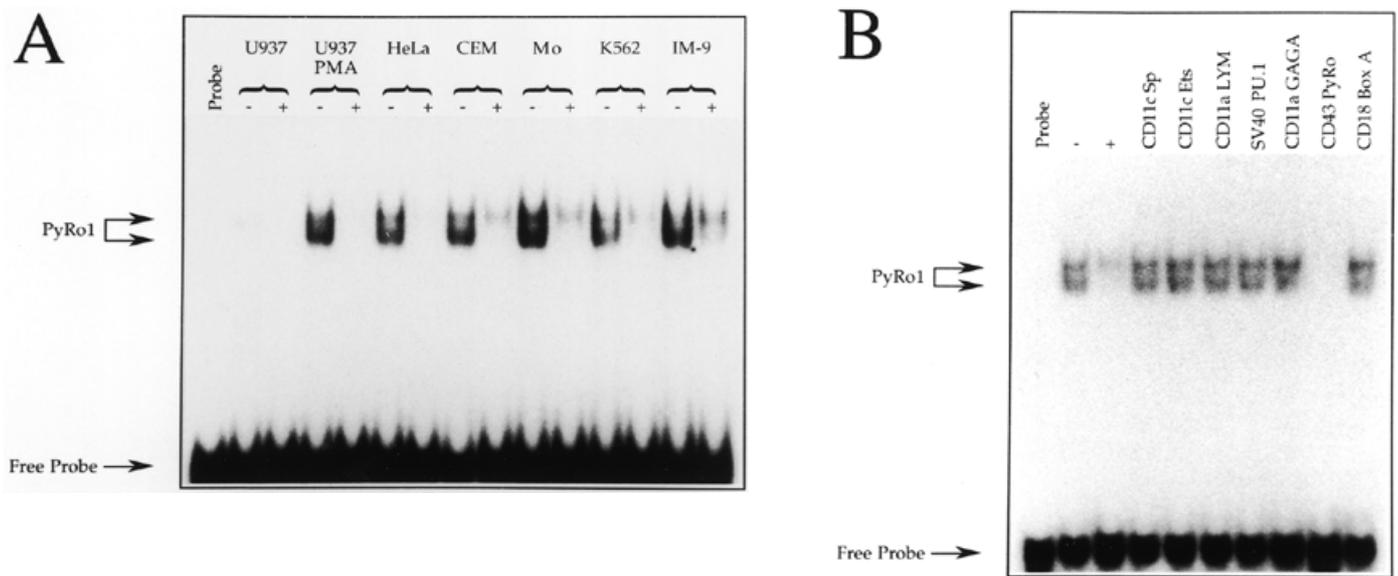


Figure 8. EMSA analysis of the nuclear proteins which interact with the $-128/-110$ region of the CD11c gene promoter. (A) A radiolabeled DNA fragment, CD11c PyRo, containing the $-128/-110$ region of the CD11c gene, was incubated with no nuclear extract (Probe) or nuclear extracts prepared from U937 cells, U937 cells induced for 24 h with PMA, HeLa cells, CEM cells, Mo T lymphoblastoid hairy cells, K562 cells or IM-9 cells. Binding reactions were performed either in the absence (–) of unlabeled specific competitor DNA or in the presence (+) of a 400-fold molar excess of unlabeled CD11c PyRo. (B) Binding reactions employing Mo nuclear extracts performed in the absence (–) or presence (+) of a 250-fold molar excess of unlabeled CD11c PyRo or the following unlabeled competitor DNA fragments: CD11c Sp and CD11c Ets, which span regions of the CD11c gene implicated in binding Sp1 and members of the ets family, respectively (72–74); CD11a GAGA, representing an MS-2-binding site within the CD11a gene (43); SV40 PU.1, known to bind the ets transcription factor PU.1 (49); CD11a LYM, which interacts with GS-1, GS-2, GS-3 and GS-4 (43); CD18 Box A, which binds ets proteins related to PU.1 and GABP (47); and CD43 PyRo, representing the +18/+39 region of the CD43 gene which interacts with PyRo1 and PyRo2 (Fig. 5). The position of the protein–DNA complex, PyRo1, is marked with an arrow, as is the position of the protein-free DNA probe.

oligonucleotides representing three of these regions were synthesized and found to effectively inhibit the interaction of PyRo1 with the sense strand of the CD43 gene (Fig. 10). This finding suggests that in addition to the CD11c and CD43 genes PyRo1 also interacts with those encoding CD11a, CD11b and CD11d.

DISCUSSION

We have identified a region spanning nucleotides +18 to +39 within the CD43 promoter which *in vitro* is hypersensitive to cleavage by nuclease S1. Such hypersensitive regions often contain elements which regulate gene expression and this proved to be the case with the CD43 gene. Deletion of the hypersensitive region almost completely abolishes CD43 promoter activity in U937 monocytic cells, CEM and Jurkat T lymphocytic cells and K562 cells, which are capable of differentiating along either the erythroid or megakaryocytic pathway. Not only is the hypersensitive region necessary for CD43 promoter activity in these cell types but it is also sufficient to induce from basal levels the activity of the SV40 promoter. Therefore, the hypersensitive region of the CD43 gene contains a complement of *cis*-acting elements with the capacity to augment the transcriptional activity of a heterologous promoter in cell lines representing a range of hematopoietic lineages. However, it is of note that this capacity is much greater in K562 and U937 cells, which are representative of the myeloid lineage, than in CEM and Jurkat cells, which are

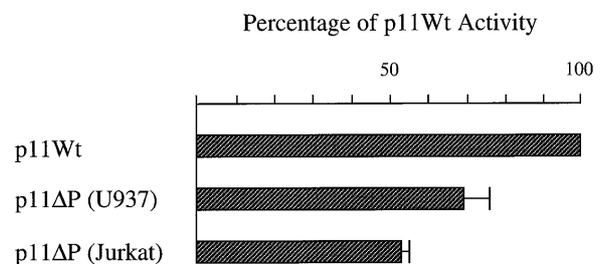


Figure 9. Contribution of the PyRo1 binding site to transcriptional activity of the CD11c promoter. The wild-type CD11c promoter spanning nucleotides -128 to $+36$ is present in the luciferase expression construct p11Wt, while p11ΔP represents the specific removal of the PyRo1-binding site extending from nucleotides -128 to -110 . The levels of luciferase gene activity in PMA-treated U937 and Jurkat cells corrected for transfection efficiency and after division by the background activity conferred by the control plasmid pATLuc are expressed as histograms. The level of expression of p11Wt is assigned an arbitrary value of 100% and the expression level conferred by p11ΔP is displayed as a proportion of this value. Each histogram represents the mean \pm SD of three independent transfection experiments.

representative of the lymphocytic lineage. EMSA analysis indicates that the hypersensitive region interacts with two nuclear factors, PyRo1 and PyRo2. PyRo2 is restricted in its DNA-binding capacity to U937 cells, suggesting that it

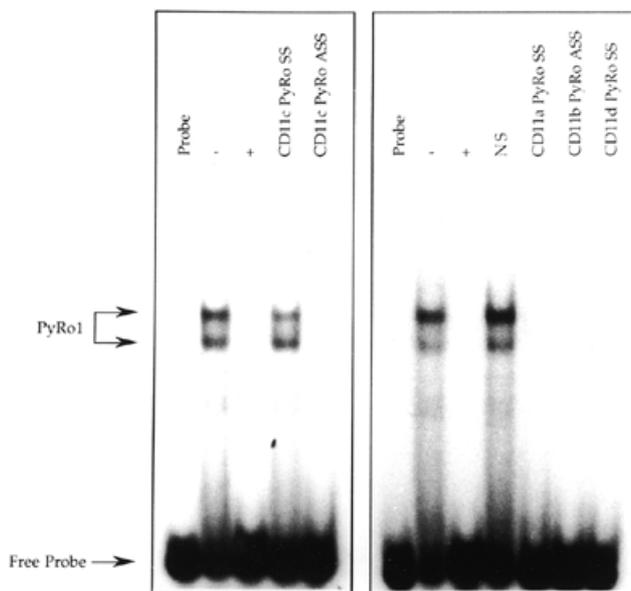


Figure 10. EMSA analysis demonstrating that PyRo1 interacts with sequences within the sense strands of the CD11a and CD11d gene promoters and the antisense strands of the CD11b and CD11c promoters. (Left) Radiolabeled CD43 PyRo SS, representing the sense strand of the +18/+39 region of the CD43 gene, was incubated with no nuclear extract (Probe) or a nuclear extract prepared from U937 cells induced for 24 h with PMA. Binding reactions were performed in the absence (-) or presence (+) of a 250-fold molar excess of unlabeled CD43 PyRo SS or in the presence of unlabeled CD11c PyRo SS or CD11c PyRo ASS, which represent the sense and antisense strands, respectively, of the -128/-110 region of the CD11c gene promoter. (Right) EMSA analysis performed as described for the left panel except that binding reactions were also performed in the presence of a 250-fold molar excess of the non-specific single-stranded oligonucleotide NS-SS (NS) or unlabeled CD11a PyRo SS, CD11b PyRo ASS or CD11d PyRo SS. These single-stranded oligonucleotides represent a sense strand sequence within the CD11a gene, an antisense sequence within the CD11b gene and a sense strand sequence within the CD11d gene, respectively.

influences CD43 expression in a manner unique to monocytic cells. In contrast, PyRo1 is expressed in a range of CD43-positive cell lines, including those representing megakaryocytes, lymphocytes and pre-erythroid/pre-megakaryocytic cells. PyRo1 is also expressed in HeLa epithelial cells which are CD43-negative. This observation suggests that PyRo1 does not play a role in restricting CD43 gene expression to hematopoietic cells. However, it remains possible that cell-specific mechanisms which fail to be reproduced in EMSA analysis contribute to the functional activity of PyRo1. Unlike other cell types which exhibit constitutive expression of PyRo1, U937 cells, which are committed to the monocytic lineage, exhibit PyRo1 DNA-binding activity only when they undergo differentiation. The inference from this expression profile is that PyRo1 is important to constitutive expression of the CD43 gene in many cell types, but in monocytic cells it plays a modulatory role. However, if such a role does exist in monocytes it is not manifest in the context of the -2/+99 promoter transfected into U937 cells. In this system mutations which abolish PyRo1 binding have no effect on promoter activity. This is also the case in CEM T lymphocytic cells. A possible explanation for these observations

is that the functional activity of PyRo1 in U937 and CEM cells is dependent upon chromatin structures not reproduced in extrachromosomal plasmids. Alternatively, PyRo1 may function in U937 and CEM cells through cooperation with a factor dependent on *cis*-acting elements lying outside the -2/+99 region. Finally, it is possible that the mechanisms by which PyRo1 influences expression of the CD43 gene were lost during the transformation process which gave rise to the U937 and CEM cell lines. The lack of PyRo1 function in CEM cells is reflected in the low level of transcriptional induction conferred in this cell line by repeats of its binding site. That these same repeats confer a substantial degree of induction in U937 cells is likely the result of the binding of PyRo2, which is specifically expressed in this cell line. In contrast to U937 and CEM cells, abolition of PyRo1 binding in Jurkat and K562 cells markedly impairs the transcriptional activity of the CD43 promoter. This indicates that in these cell lines PyRo1 plays a necessary functional role. Within K562 cells multiple binding of PyRo1 immediately upstream of a heterologous promoter is also sufficient to induce transcriptional activity. However, within Jurkat cells this ability is at a level equivalent to that in CEM cells, where mutation analysis has established that PyRo1 is not functional. Therefore, in the context of the intracellular environment provided by Jurkat cells PyRo1 appears necessary but not sufficient to induce transcription. This difference in function between Jurkat and K562 cells and also the difference in function within these cells compared to U937 and CEM indicates that the mechanisms by which PyRo1 influences gene expression are markedly cell-specific.

PyRo1 and PyRo2 may be novel factors since their interaction with the CD43 promoter fails to be competed with binding sites for the transcription factors NF- κ B, AP-1, AP-2, AP-3, Oct-1, NF-1, PU.1, Sp1 and CREB. That a binding site for the ets transcription factor PU.1 fails to interact with either PyRo1 or PyRo2 is particularly striking. This is so because the hypersensitive region of the CD43 promoter contains in the antisense DNA strand two GGAA sequences which conform to the consensus found at the core of all ets-binding sites (53).

Since the binding site for PyRo1 and PyRo2 within the CD43 promoter is hypersensitive to digestion by nuclease S1, this region clearly has the capacity to exist in a single-stranded conformation. This raised the possibility that PyRo1 and/or PyRo2 might interact with single-stranded DNA. Our analysis has established that PyRo1 does indeed interact with such DNA. Specifically, we have shown that PyRo1 interacts with the sense but not the antisense strand of the +18/+39 region within the CD43 promoter and that PyRo1 binding is largely lost when these two strands are annealed to produce double-stranded DNA.

In addition to the sense strand of the CD43 gene, PyRo1 also interacts with the antisense strand of the -128/-110 region of the CD11c gene. Comparison of the CD43 and CD11c binding sites reveals that both contain the sequence CCCAC upstream of a run of four cytosine residues. Within the CD43 binding site these motifs are separated by 7 nt, while in the CD11c site they can be mapped either adjacent to one another or separated by one nucleotide. Similar adjoining motifs which bind PyRo1 are also found within the CD11a, CD11b and CD11d genes (Fig. 11). Mutation analysis of sequences within the CD43 gene indicates that *in vitro* the CACCC motif plays little or no role in PyRo1 binding, while the CCCC motif is critical, therefore

CD43 Sense	5' -GGG <u>CCCAC</u> TTCCCTT <u>TCCCCT</u> TG-3'
CD11a Sense	5' -CAAAT <u>CCCAC</u> GGGCCTCCTGACG-3'
CD11b Anti-Sense	5' -GCCTG <u>CCCAC</u> CCTT <u>CCTCCC</u> AGCTT-3'
CD11c Anti-Sense	5' -CACACGCC <u>CCCAC</u> CCCCCA-3'
CD11d Sense	5' -TCCTA <u>CCCAC</u> TGTG <u>CCCTC</u> TC-3'

Figure 11. Nucleotide sequences within the CD11 and CD43 genes which interact with PyRo1. The nucleotide sequences depicted are those of the oligonucleotides which interact with PyRo1 (Fig. 10). Sequences are aligned relative to the motif CCCAC common to all the oligonucleotides. This motif is underlined, as is a second located downstream of the first also shared by the PyRo1-binding sites. The second motif consists of a run of five pyrimidine residues which contains the sequence CCCC or CCTCC. Within the CD11b and CD11d genes these sequences overlap, extending the motif to seven residues. Since within the CD43 gene the second motif can be read as either TCCC or CCCCT, both possibilities are underlined.

likely participating in direct protein contacts. However, *in vivo* the CACCC motif may also be critical, not by directly contacting PyRo1 but by providing a local single-stranded DNA conformation in which binding is possible. In this regard it is of interest that in each of the PyRo1-binding sites we have identified the CACCC motif could be involved in the formation of cruciform, slippage or triple helix structures, the single-stranded loop regions of which would contain the CCCC motif. The PyRo1 binding site within the CD43 gene, for example, lies in a region capable of forming all three single-stranded structures. Within the CD11 genes no inverted repeats which might form the stems of a cruciform flank the PyRo1 binding sites. However, these PyRo1 sites do contain homopyrimidine mirror repeats which might form triple helices (54). Such H-DNA structures would contain a non-paired pyrimidine-rich loop capable of PyRo1 interaction. Another possibility is that direct repeats associated with the CD11 PyRo1 sites could form slippage structures, which again would contain single-stranded regions (54).

A number of mammalian transcription factors have been identified which interact with single-stranded DNA. Those which, like PyRo1, interact with pyrimidine-rich sequences include the Y box family, PTB, ssPREB, CBF-A, STR, hnRNP K, FBP, the MSSP family, mARS bpG, Puf, VACsBF1, ssPyrBF and MyEF-3 (55–70). However, none of these factors both act as an activator and exhibit the same pattern of electrophoretic mobility and tissue-specific expression as PyRo1.

The sites we have identified which interact with PyRo1 often overlap those which bind other factors. This suggests that PyRo1 may either cooperate or compete with these factors. Within the CD11b promoter the PyRo1 binding site overlaps that of the nuclear protein MS-2, which exactly mirrors PyRo1 in its pattern of cell-specific and developmental expression (43,71). The importance of this PyRo1/MS-2 site is apparent from its mutation, which causes a >50% drop in the activity of the CD11b promoter in PMA-treated U937 cells (45). In the context of the CD11c promoter the –128/–110 region which interacts with PyRo1 is located within the sequence spanning nucleotides –135 to –99 which binds Sp1 and Sp3 (72–75). These two factors interact with double-stranded DNA. It is possible, therefore, that PyRo1 and Sp1/Sp3 binding to the

CD11c promoter are mutually exclusive events. An analogous situation may well exist within the CD43 promoter, where the PyRo1 binding site overlaps that of PyRo2 which appears only to bind double-stranded DNA. A logical conclusion of this speculation about the relationship of the binding of PyRo1 to that of other factors is that DNA secondary structure may play an important role in controlling both CD43 and CD11 gene expression.

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