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Proximal Promoter of the Murine Syndecan-1 Gene Is Not Sufficient for the Developmental Pattern of Syndecan Expression in B Lineage Cells

Carmelo P. Volpe,[1] Anna Lundgren,[1] Alar Aints,[1,2] Abdalla J. Mohamed,[1] Panu Jaakkola,[5] Birger Christensson,[3] Gösta Gahrton,[2] Markku Jalkanen,[5] C.I. Edvard Smith,[1,3,4] and M. Sirac Dilber[1,2]*

[1]Center for Biotechnology, Department of Biosciences, Karolinska Institute, NOVUM, Huddinge, Sweden
[2]Department of Medicine, Huddinge Hospital, Karolinska Institute, Huddinge, Sweden
[3]Department of Clinical Immunology, Microbiology, Pathology and Infectious Diseases, Huddinge Hospital, Karolinska Institute, Huddinge, Sweden
[4]Gene Therapy Research Center, Clinical Research Center, Huddinge Hospital, Karolinska Institute, NOVUM, Huddinge, Sweden
[5]Turku Center for Biotechnology, BioCity, Turku, Finland

Syndecan-1 (CD138) is a cell membrane proteoglycan that binds extracellular matrix components and various growth factors. The role of syndecan-1 in the control of cell growth and morphology has been illustrated by its altered expression in hematological malignancies such as multiple myeloma as well as some solid tumors. It has been reported that the expression of syndecan-1 in cells of the B lineage is developmentally regulated such that pre-B cells and plasma cells express syndecan-1 while mature B cells do not. Thus, we investigated whether the proximal promoter region of the murine syndecan-1 promoter was able to confer the observed on–off–on expression of syndecan-1 in cells of the B lineage as they develop from pre-B cells to plasma cells. Experiments carried out using deletion mutants of the proximal promoter cloned upstream of the CAT reporter gene transfected into murine cell lines, representing the above stages of B-cell development, such as BA/F3 (pro-B cell), 70Z/3 (pre-B cell), 2PK3 (late mature B cell), and MPC-11 (plasma cell), showed detectable levels of CAT expression. The WEHI-231 (mature B cell) cell lines did not show detectable levels of CAT reporter activity. The strong levels of expression were observed with a fragment of the proximal promoter spanning the region from −365 to −95 (from the translation start point). Hovewer, Northern analysis of RNA obtained from the five murine B-cell lines, representing various stages of B-cell development, showed that the 70Z/3, MPC-11 but not BA/F3, and 2PK3 cells expressed detectable levels of syndecan-1 mRNA. By FACS analysis, using a rat anti mouse syndecan-1 antibody, syndecan-1 expression on the cell surface was found to correlate with the observed mRNA expression patterns in these cell lines. Our results indicate that the proximal promoter of the murine syndecan-1 promoter is not sufficient for the observed developmental pattern of syndecan expression in B cells. Am. J. Hematol. 67:20–26, 2001.

Key words: syndecan-1 promoter; B cells; multiple myeloma; proteoglycan; gene therapy

INTRODUCTION

Syndecan-1 is a cell membrane proteoglycan that binds both to components of the extracellular matrix and to members of the heparin binding growth factors. Its core protein consists of an extracellular domain containing glycosaminoglycan chains, a highly conserved transmembrane domain, and a short cytoplasmic domain that is related among all four known members of the syndecan family [1]. Syndecan-1 is expressed on pre-B cells within the bone marrow, lost as cells mature (before re-

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*Correspondence to: M. Sirac Dilber, M.D., Ph.D., Karolinska Institute, Hematology, KFC, NOVUM, S-141 86, Huddinge, Sweden.
E-mail: Sirac.Dilber@medhs.ki.se

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lease from the marrow), is absent on circulating and peripheral B cells, and re-expressed on plasma cells [2].

The role of syndecan-1 in the control of cell growth and morphology is illustrated by its altered expression in some solid tumors [3,4] as well as in hematological malignancies such as multiple myeloma [5,6]. It has been shown that syndecan-1 is expressed by most malignant plasma cells and that cell surface syndecan-1 mediates both cell–cell and cell–extracellular matrix adhesion and inhibits the invasion of cells through type I collagen matrix, indicating that syndecan-1 may be important in regulating the progression of multiple myeloma [6]. It has also been reported that syndecan-1 expression is restricted to myeloma tumors with a well-differentiated, i.e., less aggressive, phenotype [5].

The molecular mechanisms responsible for the regulation of syndecan expression are beginning to be explored. The complete structure and nucleotide sequence of the murine syndecan-1 gene, including a 9.5-kb fragment of its upstream region, has been previously characterized [7–9]. Major regulatory elements for syndecan-1 expression in epithelial cells have also been well documented [9], but there is to our knowledge no information of the promoter region regulating the expression of syndecan-1 during B-cell development. The unique expression pattern of syndecan-1 (on–off–on) during B lymphocyte development plays an important role in the pathobiology of hematological malignancies, such as multiple myeloma. We investigated the murine syndecan-1 promoter activity in the B-cell lineage with the purpose of identifying fragments suitable for use in gene therapy vectors to confine the expression to mature plasma cells.

Deletion mutants of the proximal promoter of syndecan-1 cloned upstream of the CAT (chloramphenicol acetyltransferase) reporter gene were transfected into murine cell lines representing different developmental stages from pro-B cells to plasma cells. CAT assay did not properly correlate FACS, Northern blot, or intracellular staining of syndecan-1. Our results indicate that the proximal promoter of the murine syndecan-1 gene is not sufficient for the observed developmental pattern of expression in murine B and plasma cells and therefore is not feasible for use in gene therapy vectors.

MATERIALS AND METHODS

Plasmid Constructs

The following clones covering the murine syndecan-1 proximal promoter region were used in this study [9]: p1.0CAT (−1,023 to −137), p492CAT (−492 to −95), p365CAT (−365 to −95), p326CAT (−326 to −95), and p289CAT (−289 to −95). The translational start site was numbered as +1 as described previously [9]. In addition, a p351CAT (−351 to −95) construct was created using the following upstream primer (5′-dTTGC**GCATGC**ACCCAGGG-3′) containing an *Sph*I site (in bold) and the following downstream primer (5′-dTTGC**TCTAGA**CTTTGCTG-3′) containing a *Xho*I site (in bold). Approximately 500 ng of each primer was mixed with 65 ng of the p1.0CAT plasmid construct, and polymerase chain reaction was performed using standard reagent concentrations using the *Pfu* thermostable enzyme. An initial denaturation step was performed at 94°C for 1 min, and then 32 cycles were performed at 94°C, 48°C, and 72°C for 30 sec at each temperature followed by 5 min at 72°C. The resulting PCR reaction was subjected to restriction enzyme digestion with *Xho*I and *Sph*I before agarose gel electrophoresis. The 255-bp fragment was identified, purified using QIAquick (Qiagen, Hilden, Germany), and after quantification ligated into *Xho*I/*Sph*I-digested pBasicCAT (Promega, Madison, WI). After transformation into JM109, transformants were selected and putative recombinants were identified by qualitative restriction digestion. The identified clones were then sequenced to confirm their identity.

Cell and Culture Conditions

Murine pro-B BA/F3 [10] (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ]: ACC 300) cells were cultured in RPMI 1640 medium (Life Technologies Inc., Paisley, Scotland) containing 10 ng/ml murine IL-3 (Genzyme, Cambridge, MA), 100 μg/ml streptomycin (Life Technologies Inc.), 100 U/ml penicillin (Life Technologies Inc.), 2 mM L-glutamine, and 10% fetal bovine serum (Life Technologies Inc.). Murine pre-B 70Z/3 [11] (ATCC: TIB-158) cells were cultured in RPMI 1640 medium (Life Technologies Inc.) containing 0.05 mM 2-mercaptoethanol, 100 μg/ml streptomycin (Life Technologies Inc.), 100 U/ml penicillin (Life Technologies Inc.), 2 mM L-glutamine, and 10% fetal bovine serum (Life Technologies Inc.). Murine B-cell lymphoma WEHI-231 [12] (ATCC CRL-1702) cells were cultured in Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose (Life Technologies Inc.) containing 0.05 mM 2-mercaptoethanol, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% fetal bovine serum. Murine B-cell lymphoma 2PK-3 [13] (ATCC TIB-203) cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies Inc.) containing 0.05 mM 2-mercaptoethanol, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 10% fetal bovine serum. The murine plasma cell line MPC-11 [14] (ATCC: CCL-167) was cultured in Dulbecco's Modified Eagle's Medium (Life Technologies Inc.) containing 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, and 20% horse serum. NIH-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium,

100 μg/ml streptomycin (Life Technologies Inc.), 100 U/ml penicillin (Life Technologies Inc.), and 10% fetal bovine serum (Life Technologies Inc.). All cell lines were cultured at 37°C in 5% CO_2.

Transfection Experiments

Cells were cultured in fresh medium, 24 hr prior to transfection, at a density of 5×10^5 cells/ml. Cells were prepared for transfection by centrifugation and resuspension at a cell density of 2×10^7cells/ml in culture medium. Approximately 10^7 cells were exposed to a single pulse of 320 V (BA/F3), 360 V (70Z/3), 300 V (2PK3), 330 V (WEHI-231), and 380 V (MPC-11) at a capacitance of 960 μF for each cell line using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). Plasmids used for transient transfection experiments were isolated using the Qiagen Plasmid Maxi Kit (Qiagen) and quantified by spectrophotometry. For each transfection 10 μg of the CAT reporter construct was co-transfected with 5 μg of a β-galactosidase construct (containing a human CMV promoter). Following transfection, the cells were incubated for 10 min at room temperature before being transferred to 9.5 ml of culture media. Cells were harvested 48 hr after transfection for determination of CAT and β-galactosidase activity.

CAT and β-Galactosidase Assays

Cell harvest was performed by first washing the cells in 0.25 M Tris-HCl, pH 7.8, and then resuspending them in 100 μl 0.25 M Tris-HCl, pH 7.8. Cells were disrupted by five cycles of 1 min freezing in liquid nitrogen followed by 1 min thawing at 37°C. To separate protein from cell debris, samples were centrifuged and the supernatant was collected. Determination of protein concentration was done with Bio-Rad Protein Assay (Bio-Rad) based on the method of Bradford using bovine serum albumin (Promega) as a standard. CAT assays were carried out according to Gorman [15]. CAT conversion was assayed by thin layer chromatography (TLC aluminium sheets, silica gel 60 F_{254}, Merck) using chloroform/methanol (95:5) as a solvent. An X-ray film was exposed to the chromatogram at room temperature until a clear signal could be detected. To monitor transfection efficiencies, a β-galactosidase assay was performed according to Hall [16] and the CAT values were normalized to these transfection efficiencies.

RNA Isolation and Northern Blot

Approximately (1–3) $\times 10^6$ cells were washed with PBS once prior to RNA extraction using TRIzol reagent (Life Technologies Inc.) and the yield quantitated by spectrophotometry at 260 nm. Total RNA (30 μg) was subjected to electrophoresis on a 1% agarose-formaldehyde gel [17] and washed in 10× SSC for 30 min, prior to being transferred to Hybond-N membrane (Amersham Life Science Ltd., Buckinghamshire, England) following the method of Thomas [18]. Blocking of the membrane was carried out with PHB (50% formamide, 0.1% SDS, 5× SSC, 200 μg/ml sonicated salmon sperm DNA, 5× Denharts [0.1% Ficoll type 400, 0.1% polyvinyl pyrrolidone, and 0.1% BSA fraction V] and 0.005% pyrophosphate tetrasodium) for 3 hr at 42°C. A gel-purified 2-kb *Eco*RI fragment of the murine syndecan-1 cDNA (PM-4) [Sauders, 1989 #23] was radiolabeled with [α-^{32}P]dCTP (Amersham Life Science Ltd.) using an Oligo labeling kit (Pharmacia Biotech, Uppsala, Sweden). Unincorporated nucleotides were removed using Nick spin columns (Sephadex G50 Fine; Pharmacia Biotech). Membranes were placed in fresh PHB, and the radiolabeled DNA was allowed to hybridize overnight at 50°C. Membranes were washed three times in 2× SSC, 0.1% SDS for 10 min at room temperature and once in 0.2× SSC, 1% SDS at 69°C. The membrane was exposed for 1 week on a Fuji BasIII imaging plate and read with a Fujix Bas 2000 Phosphoimager (Fuji Photo Film Co. LTD., Tokyo, Japan). Image processing was done using Bas 2000 Image File Manager 3.0 on a Sun SparcStation.

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared from BA/F3, 70Z/3, WEHI-231, 2PK-3, MPC-11, and 3T3 cells following the method of Dignam and co-workers [19]. EMSA analysis was based on the method of Dent and Latchman [20]. Pre-binding reactions were carried out in a total volume of 20 μl of 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 4% Ficoll, 0.5 mM DTT, 50 mM KCl, 1.5 μg poly d[I·C] (Boehringer Mannheim Scandinavia AB, Bromma, Sweden), and 2 μg of dialyzed protein extract. Pre-binding reactions were placed on ice for 15 min prior to the addition of 10^4 cpm of [α-^{32}P]dCTP fill-in labeled DNA probe (unincorporated nucleotides were removed using Sephadex G50 Fine Nick spin columns [Pharmacia Biotech]) and incubated on ice for an additional 45 min. Complexes were resolved by electrophoresis at 10 mA through a 4% polyacrylamide gel using 22.5 mM Tris-borate, 0.5 mM EDTA (pH 8.0) buffer. Indicated amounts of unlabeled specific competitors were added to the pre-binding reactions and were used with the indicated probes to distinguish sequence-specific interactions. Consensus double-stranded binding site oligonucleotides for Sp1 (5′-dATTCGATCGGGGCGGGGCG AGC-3′) and NF-κB (5′-dAGTTGAGGGGACTTTC-CCAGGC-3′) were used in the analysis.

FACS Analysis

Flow cytometric analysis was performed according to standardized procedures using a FACScan (Becton-Dickinson Immunocytometry Systems [BD], San Jose, CA). Data acquisition and analysis were carried out with

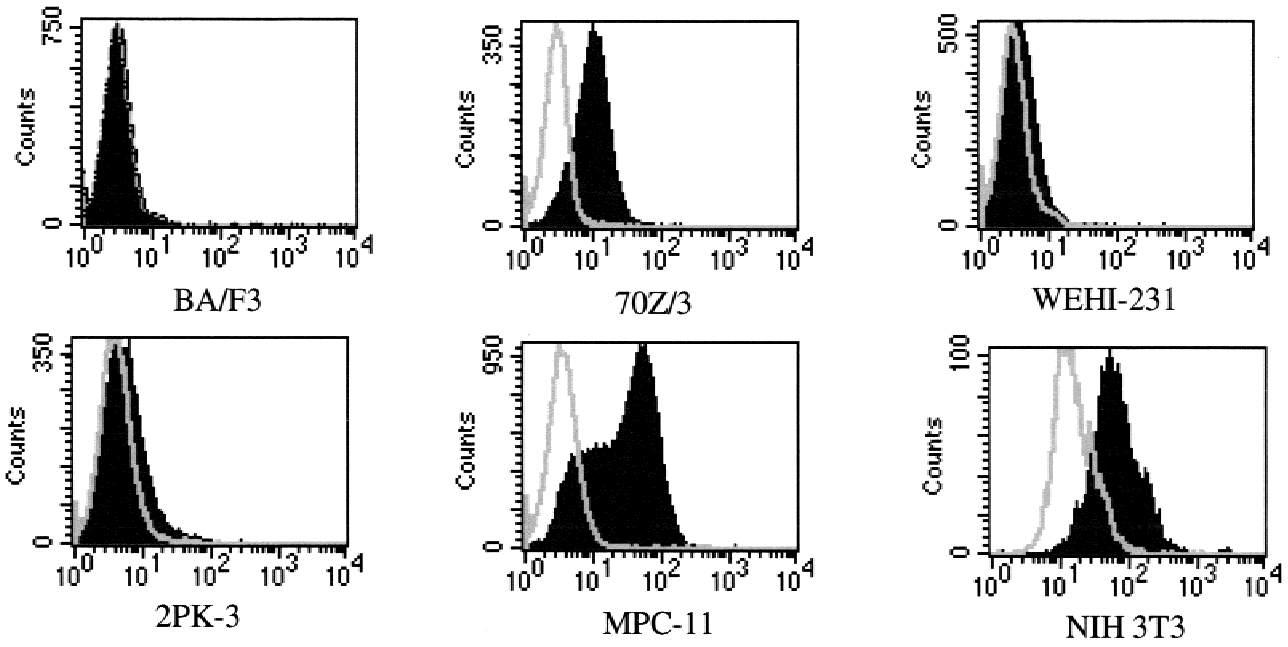

Fig. 1. Flow cytometric analysis of syndecan-1 expression on the cell surface. The cell lines were stained in parallel with either a biotinylated monoclonal antibody to murine syndecan-1 or a matching isotype control. The excess fluorescence (median value) seen with the syndecan-1 antibody was 1.07 for BA/F3, 1.28 for WEHI-231, 1.37 for 2PK-3, 3.52 for 70Z/3, 4.07 for NIH 3T3, and 22.86 for MPC-11. The syndecan-1 expression observed on the cell surface by flow cytometry was also observed by immunohistochemistry. In none of the cell lines was intracellular syndecan-1 expression observed in the lack of surface staining.

CellQuest software (BD). The antibody for the detection of mouse syndecan-1 was a biotinylated rat anti-mouse CD138 (clone 281-2) obtained from Pharmingen (San Diego, CA). Cells were washed in phosphate-buffered saline (PBS), and 100 μl of cell suspension containing 5×10^5 cells was incubated at room temperature for 30 min with 0.1 μg/ml of the biotinylated antibody. After being washed twice, FITC-conjugated streptavidin (Dakopatts, Glostrup, Denmark) was added to the cells at a 1/50 dilution and incubated for 30 min in the dark. After the cells were washed, propidium iodide (PI, Sigma St. Louis, MO) was added at 2 μg/ml and the cells were analyzed within 20–30 min. For each sample at least 10^4 cells were acquired using log-amplified fluorescence and linearly amplified side and forward scatter signals. The samples were analyzed by setting appropriate FSC/SSC gates around the viable cell population using back-gating on PI negative cells. The shift in FITC staining was visualized by histogram analysis. Unstained cells and cells stained with streptavidin-FITC alone served as negative controls. The median linear value for FITC intensity of the syndecan-1 stained cells divided by the corresponding value for the negative control gave the excess staining for syndecan-1.

RESULTS

Syndecan-1 Expression

It has been previously reported that the level of syndecan-1 cell surface expression correlates with the stage of differentiation of B cells. Thus the presence or absence of syndecan-1 on the surface of the cell lines used in this study was investigated through FACS analysis using rat anti-murine syndecan-1 (CD138) antibody (Fig. 1). From Fig. 1 it can be seen that pre-B cells (70Z/3), plasma cells (MPC-11), and fibroblasts (3T3) express large quantities of syndecan-1 protein. However, in contrast, the pro-B cell (BA/F3) and mature B cell (WEHI-231) do not appear to express syndecan-1 on their surface. The mature B cell line 2PK-3 did not express significant amounts of cell surface syndecan-1. Gene expression can be regulated at many levels including translation. Thus, to determine if transcriptional regulation was occurring within these cell lines, Northern blot analysis was performed using a 2-kb *Eco*RI fragment of PM-4 cDNA clone for syndecan-1 [Sauders, 1989 #23] (Fig. 2). Figure 2 shows that MPC-11 and 3T3 cells express large quantities of syndecan-1 RNA while the pre-B cell line 70Z/3 expresses a much lower level of syndecan-1 mRNA. These results correlate well with the FACS analysis, which indicated that 70Z/3 and MPC-11 express syndecan-1 protein on their surface. In addition, Fig. 2 shows that no expression of syndecan-1 could be detected in WEHI-231 or BA/F3, again correlating well with the data from the FACS analysis. Although the late mature B cell line 2PK-3 showed a weak expression of syndecan-1 on their surface and in the CAT activity, mRNA was not detected by a Northern blot. Syndecan-1 mRNA may still be present in very low concentration, under detection limits. In

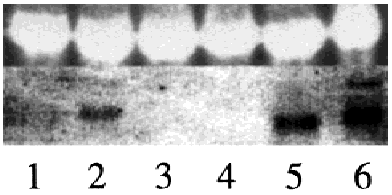

Fig. 2. Northern blot analysis of the murine syndecan-1 expression in B lineage cell lines. (Upper panel) 18S RNA band stained with ethidium bromide on the filter used for probing the syndecan-1 mRNA. (Lower panel) Northern analysis of total RNA isolated from the cell lines indicated and probed with a 2-kb fragment of the murine syndecan-1 cDNA. Cell lines: 1, BA/F3; 2, 70Z/3; 3, WEHI-231; 4, 2PK-3; 5, MPC-11; 6, NIH 3T3.

both the FACS analysis and the Northern blot, NIH 3T3 cells were analyzed for their ability to express syndecan-1. In both analyses, syndecan-1 was detected. This cell line has previously been reported to express syndecan-1 and was used as a positive control in these experiments.

Promoter Activity in B Cells

While the murine syndecan-1 promoter has been previously investigated in epithelial cells and fibroblasts [9], the promoter activity in B cells has not. Deletion constructs of the syndecan-1 promoter, namely p1.0CAT, p492CAT, p365CAT, p351CAT, p326CAT, and p289CAT (Fig. 3A), were used to investigate the role of these promoter regions in the expression of syndecan-1 during B-cell differentiation. Figure 3 shows results of this analysis. It can be seen that the overall level of CAT activity observed was low and thus was a limiting factor in this investigation. Analysis of pro-B (BA/F3) and plasma cells (MPC-11) showed that all constructs were capable of driving the CAT reporter construct. Closer inspection of the constructs showed that the high level of expression was observed with the p351 and p365 constructs. Unexpectedly, pro-B cell (BA/F3), which had showed essentially no endogenous syndecan-1 expression by Northern and FACS analysis, showed the highest levels of syndecan-1 promoter-driven CAT expression among the B cell lines tested. To determine possible negative regulatory elements in the proximal promoter region acting in the mature B cell (WEHI-231), an analysis was carried out using progressively shorter promoter regions. However, it was not possible to detect any CAT reporter expression above background levels. Thus, together these results still suggest that the minimal proximal promoter of the syndecan-1 gene is not sufficient for the developmentally regulated expression of syndecan-1.

EMSA Analysis

Previous analysis of the proximal promoter region of the syndecan-1 gene had demonstrated that members of the Sp family of transcription factors were capable of binding to the GC/GT boxes identified by sequence analysis. Using sequence analysis of this region we identified an NF-κB consensus sequence (Fig. 3A). Using competitive EMSA we investigated the ability of either the Sp family members or the NF-κB, from B-cell extracts, to bind to their respective consensus binding sites in the proximal promoter region. A 146-bp long (*Bln*I/*Pst*I) fragment was isolated from the proximal promoter and cleaved with *Ava*I to generate two fragments of 81 bp and 65 bp, which were used as probes. Competitions were performed by using 100-fold excess of unlabeled consensus Sp1 or NF-κB oligonucleotide either alone or in combination for each extract.

From Figure 4 it can be seen that in each case the shifted complexes could be competed out with the consensus Sp-1 binding site, showing that Sp family members were present in these extracts and were capable of binding to the syndcan-1 promoter. In contrast, it was not possible to compete out the binding using the NF-κB consensus oligonucleotide.

DISCUSSION

The pattern of syndecan-1 expression observed in B cells could be explained by at least three types of transcriptional mechanisms: First, positive regulation of syndecan-1 may occur via the binding of a factor that is present in pre-B cells and in plasma cells but which is absent in mature B cells. Second, positive regulation of syndecan-1 may occur via the binding of two different factors, one of which is selectively expressed in pre-B cells and the other in plasma cells. Thirdly, negative regulation may result from the expression of a factor in mature B cells that prevents expression of syndecan-1 in these cells. It is also possible that some regulation occurs at the level of mRNA stability. In an attempt to discriminate between these possibilities, deletion constructs were analyzed for their ability to drive syndecan-1 expression in B cells of different lineage.

Analysis of syndecan-1 promoter activity by the CAT assay showed that in pro B-cells (BA/F3), p351-492 constructs of the syndecan-1 promoter were required to achieve the highest levels of expression. In MPC-11 and 2PK-3, however, the p365 construct was singly the most active. This region has been shown to contain two GC/GT boxes [9]. These sequences are common binding sites for the Sp family of transcription factors.

During this investigation we noticed that in BA/F3 cells, the levels of CAT activity observed was manyfold higher than in any other cell line examined. This observation was at odds with the results of the Northern blot and FACS analysis, which showed no detectable syndecan-1 expression. It seems likely that the full-length endogenous promoter contains a strong negative regulatory element that was not present in the promoter constructs discussed above. In an attempt to determine if this potential negative element was located further upstream of

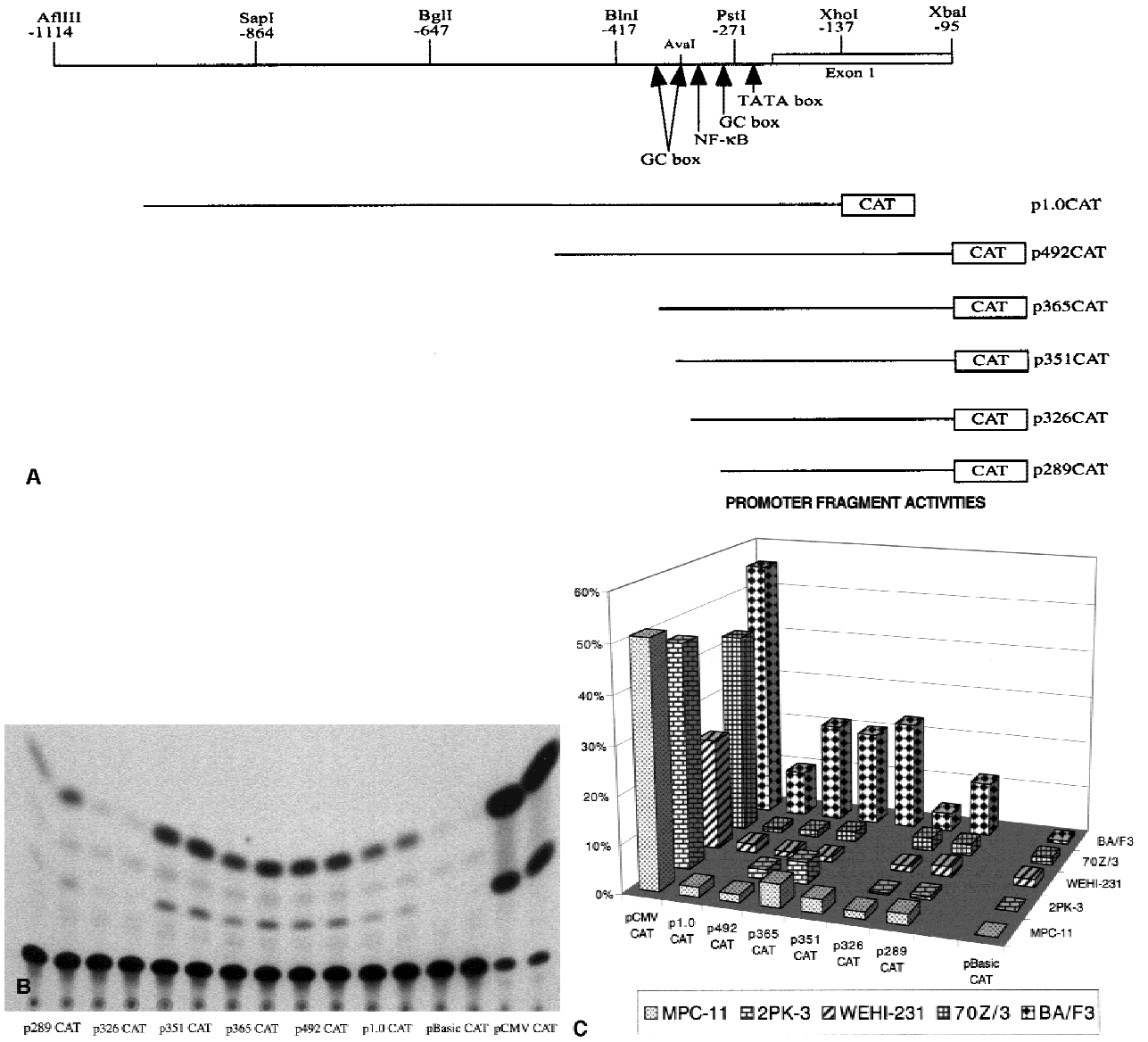

Fig. 3. (A) Schematic representation of the murine Syndecan-1 promoter showing the location of the restriction sites and consensus binding sites as identified by the Findpatterns program from the Wisconsin GCG suite of software. The deletion clones used in this study are also shown. (B) CAT assay performed on BA/F3 cells using the following constructs: 1, p289 CAT; 2, p326 CAT; 3, p351 CAT; 4, p365 CAT; 5, p492 CAT; 5, p1.0 CAT; 6, pCAT Basic (negative control); 7, pCMV CAT (positive control). (C) Graphic representation of promoter fragment activities in the cell lines studied. Gel Pro Analyzer 2.0.10 software from Media Cybernetics was used to quantitate the CAT assays, and Microsoft Excel was used to draw the graph. Bars represent the relative abundance of acetylated chloramphenicol.

the syndecan-1 promoter, additional CAT assays were carried out with a 5′ flanking sequence extending 12 kb upstream of the transcription start point. However, no significant negative effect on the CAT expression was observed, suggesting that the inhibitory sequence is not located within this region upstream of the transcription start point and thus, if it exists, may be located in an intron or 3′ of the coding gene.

It was observed that plasma cells (MPC-11) expressed very high levels of syndecan-1 surface protein and mRNA but a low level of CAT activity. It seems likely that the endogenous promoter contains a regulatory element that was not present in the promoter constructs that have been used, which may contribute to the higher expression in plasma cells.

The negative results using progressively shorter promoter sequences in the CAT analysis of the mature B-cell (WEHI-231) indicated that the region(s) required for the negative regulation of syndecan-1 expression in mature B cells either was not located in the proximal promoter or was located very close to the transcription start site. Thus, the progressive deletions did not result in loss of

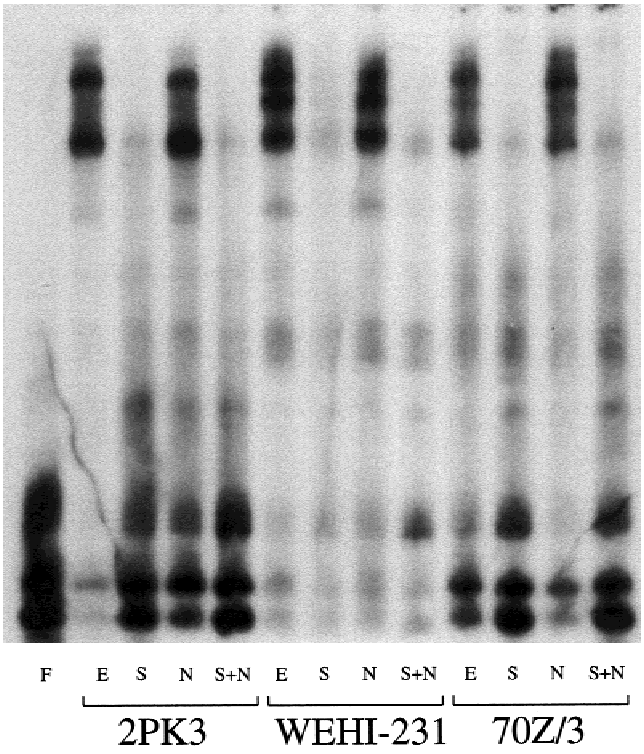

Fig. 4. Electrophoretic mobility shift assay (EMSA). F, free fragment; E, nuclear extract; S, 100-fold excess of Sp-1 one consensus binding site oligo added; N, 100-fold excess of NF-κB consensus binding site oligo added; N+S, both oligos added.

binding site for a putative negative regulator, which would have been observed as increased CAT activity. However, our results do not rule out that the expression of syndecan-1 in WEHI-231 cell line requires the binding of positive regulatory factor(s) that either are lacking or the binding site(s) of which was not present in our constructs. As noted, the region that gave the highest level of CAT expression in the syndecan-1 promoter has many binding sites for the Sp family of transcription factors. Recently it was reported that the Sp3 factor is pivotal, since it can either activate or repress transcription depending on cell type and DNA binding context [21,22]. Such a "context-sensitive" regulation could possibly explain the decreased expression of syndecan-1 in mature B cells.

In conclusion, our results indicate that the proximal promoter region does not contain the key regulatory elements for the developmental expression pattern of syndecan-1 in the B-cell lineage. The probable location for such elements is further upstream of the tested 12-kb area, introns, coding sequence, or 3′ of the gene. We consider this work to be a guideline for further investigations. Targeted expression in plasma cells and myeloma cells utilizing only the syndecan-1 proximal promoter control elements is not possible.

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