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Leukocyte-specific expression of the *pp52* (LSP1) promoter is controlled by the *cis*-acting *pp52* silencer and anti-silencer elements

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Abstract

pp52 (LSP1) is a leukocyte-specific phosphoprotein that binds the cytoskeleton and has been implicated in affecting cytoskeletal remodeling in a variety of leukocyte functions, including cell motility and chemotaxis. The expression of *pp52* is restricted to leukocytes by a 549 bp tissue-specific promoter. Here, we show that promoter fragments smaller than the 549 bp *pp52* promoter have activity in fibroblasts where *pp52* is not normally expressed. Specifically, a truncated construct (+1 to −99) functioned as a basal promoter active in leukocytes and fibroblasts. We identified two upstream regions within the 549 bp *pp52* promoter responsible for restricting *pp52* promoter activity in fibroblasts. These two regions contained a silencer (*pp52* NRE) and an anti-silencer (*pp52* anti-NRE) with opposing activities controlling *pp52* gene expression. The *pp52* NRE was active in both leukocytes and fibroblasts while the *pp52* anti-NRE was only active in leukocytes, thereby allowing *pp52* gene transcription in leukocytes but not in fibroblasts. The *pp52* NRE was localized to an 89 bp DNA segment between −324 and −235 in the 549 bp *pp52* promoter and functioned as an active silencer element in a position and orientation independent manner. The *pp52* anti-NRE was localized to a 33 bp segment between −383 and −350 of the 549 bp *pp52* promoter and acted as an anti-silencer element against the *pp52* NRE, but lacked any intrinsic enhancing activity on its own. These findings indicate that the tissue specificity of the *pp52* promoter is determined by the *pp52* anti-NRE anti-silencer which over-rides the general inhibitory activity of the *pp52* NRE silencer.

Keywords: Transcription; Gene regulation; Negative regulatory element

1. Introduction

The *pp52* gene, also isolated as *LSP1* (Jongstra et al., 1988; Jongstra-Bilen et al., 1990) and *WP34* (Kadiyala et al., 1990), encodes a 52 kilodalton leukocyte-specific intracellular phosphoprotein implicated in affecting cytoskeletal architecture and function (Jongstra-Bilen et al., 1990, 1992). All B-lineage cells, granulocytes, macrophages, and primary T cells express *pp52* mRNA (Jongstra et al., 1988; Gimble et al., 1993; Howard et al., 1994; Jongstra et al., 1994; Li et al., 1995). However, *pp52* mRNA is not expressed in some transformed murine T cell lines, and is found at extremely low levels in a subset of transformed human T cell leukemias (Jongstra et al., 1988; Jongstra-Bilen et al., 1990; Kadiyala et al., 1990). The *pp52* protein binds filimentous actin (F-actin) and co-caps with the B cell antigen receptor following surface immunoglobulin cross linking with anti-Ig antibody (Klein et al., 1990; Jongstra-Bilen et al., 1992). Through F-actin binding and membrane localization, *pp52* potentially links the cytoskeleton with the cytoplasmic face of the plasma membrane (Klein et al., 1989). Elevated *pp52* expression is implicated in the rare genetic disorder, neutrophil actin dysfunction (NAD) (Howard et al., 1994). NAD neutrophils have extended F-actin-rich surface projections, abnormal cellular morphology, and impaired motility (Howard et al., 1994, 1998). A recent study has shown that the impairment of leukocyte motility is directly related to the degree of *pp52* elevation (Li et al., 2000). Other recent findings indicate that elevated *pp52* expression levels are correlated with the distinctive

Abbreviations: anti-NRE, anti-negative regulatory element; bp, base pair(s); CAT, chloramphenicol acetyltransferase; DNase, deoxyribonuclease; ds, double stranded; EMSA, electrophoretic mobility shift assay; F-actin, filimentous actin; Ig, immunoglobulin; Inr, initiator element; NAD, neutrophil actin dysfunction; NRE, negative regulatory element

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spiky projections found in Hairy Cell Leukemia where the leukemic B cells strikingly resemble the affected neutrophils in NAD (Miyoshi et al., 2001).

Leukocyte-specific *pp52* transcription is driven by a TATA-less promoter which initiates from a single start site determined by an initiator element (Inr) (Thompson et al., 1996; Omori et al., 1997). The 549 bp *pp52* promoter is strictly tissue specific and is therefore highly active in B cells and T cells, but inactive in 3T3 fibroblasts. Duplicated Ets and Sp1 binding motifs and a single central C/EBP factor binding site are critical for tissue-specific *pp52* (−549) promoter activity (Fig. 4 and Omori et al., 1997). However, these factors are not specific to leukocytes, leaving open the question of how the strict tissue-specificity of the *pp52* promoter is determined.

Here, we describe a mechanism for regulating tissue-specific expression of the *pp52* (−549) promoter. We noted that *pp52* promoter fragments smaller than the *pp52* (−549) leukocyte-specific promoter lost tissue specificity and were expressed in fibroblasts, albeit at lower levels than in B cells. The region from −99 to +1 containing the Ets and Sp1 motifs functioned as a basal promoter in B cells and fibroblasts. Deletion series of the *pp52* (−549) promoter also revealed a region that reduced expression of the *pp52* promoter in both B cells and fibroblasts, and a region that restored activity to leukocytes but not to fibroblasts. These data suggested a silencer/anti-silencer interplay controlling *pp52* tissue-specific expression. Silencer elements are defined as *cis* -acting DNA regulatory elements that down regulate gene transcription (Chen et al., 1993; Young and Korsmeyer, 1993; Martensson and Melchers, 1994; Ernst and Smale, 1995), while anti-silencer elements have been shown to restore gene activity to pre-silencer levels by over-riding silencer activity (Stover and Zehner, 1992; Ferradini et al., 1994; Dobretsova and Wight, 1999; Izmailova and Zehner, 1999). Our results suggest that the *pp52* anti-NRE (i.e. anti-silencer) counteracts the *pp52* NRE silencer in leukocytes to support *pp52* expression in leukocytes. Since the *pp52* anti-NRE is not active in fibroblasts, it therefore cannot counteract the silencing activity of the *pp52* NRE, which restricts *pp52* expression in fibroblasts.

2. Materials and methods

2.1. Cells, DNA transfections, and CAT assays

All cell lines were propagated in RPMI 1640 supplemented with sodium pyruvate (GibcoBRL, Gaithersberg, MD), non-essential amino acids (GibcoBRL), 50 μM β-ME (Sigma Chemicals, St. Louis, MO), 5 mM glutamine (Sigma), and 5% fetal calf serum (FCS, Omega Scientific, Tarzana, CA). 1×10^7 M12 B cells were co-transfected with 5 μg of CAT constructs and 1 μg of pRSV-luciferase by the DEAE-dextran method as described (Grosschedl and Baltimore, 1985). 3-1 pre-B cell line, BW5147 T cell line, and EL-4 T cell line were transfected by electroporation as previously described (Omori et al., 1997). NIH 3T3 fibroblasts and Raw264 macrophage cell line were transfected using the calcium phosphate method (Ausubel et al., 1994) with one modification. Precipitated DNA (5 μg of CAT constructs and 4 μg of pRSV-luciferase) was added to 70% confluent monolayers in 100 mm dishes whose media had been replaced by DMEM plus 5% Transfector II (modified bovine serum (MBS), Stratagene, La Jolla, CA) immediately prior to transfection. Cells were incubated for 3 h at 37°C, 5% CO_2, washed three times with 1 × PBS, and grown in supplemented RPMI plus 5% FCS. All transfections were harvested 40–48 h after transfection. Extracts were prepared and CAT assays performed as described previously (Omori and Wall, 1993). Quantitations were done using a Molecular Dynamics Phosphorimager.

2.2. Plasmid construction and deletion mutagenesis

The *pp52* (−549) and *pp52* (−350) deletion constructs were generated using internal Xba1 and EcoR1 sites, respectively, found in the original *pp52* (−1.0 kb) construct previously described (Thompson et al., 1996). All deletion fragments were subcloned into pCAT-Basic (Promega, Madison, WI) and indicated by number of bases from the start site of transcription (+1). The *pp52* (−307), *pp52* (−293), *pp52* (−257), *pp52* (−204), and *pp52* (−99) deletions were generated by exonuclease III/mung bean nuclease (Stratagene) digestion of the *pp52* (−350) promoter fragment CAT construct. The *pp52* endogenous ATG was destroyed leaving the first methionine of the CAT gene as the translational start site. For the negative regulation studies, an 89 bp Alu1 fragment encompassing the potential silencer region was ligated to HindIII linkers and subcloned 5′ of the *pp52* (−99) promoter construct. *pp52* (−350-NRE) and *pp52* (−549-NRE) promoter constructs were generated by creating EcoRV restriction sites at −324 and −235 using the Quik-Change mutagenesis kit (Stratagene) in the *pp52* (−350) and (−549) promoter constructs, excising the intervening sequences by restriction digest, and religation of the resulting plasmid minus −324 to −235 putative silencer sequences.

2.3. Nuclear extracts and DNA-binding assays

Preparation of crude nuclear extracts from cell lines were prepared as described (Lo et al., 1991). DNase 1 protection assays were performed as described (Lo et al., 1991). DNase 1 protection probe was generated by 5′ end-labeling with [γ-^{32}P]ATP 5′ PCR primers (−473; 5′-GGCCATGACCTTGAAGCATC-3′ and −428; 5′-GAGCACACTTGGAAGCACAG-3′) and subsequent *pfu* (Stratagene) PCR cycling at 95°C, 30 s; 55°C, 1 min; 72°C, 2 min; using the *pp52* −549 plasmid construct as the template. DNase 1 protection probes were purified by StrataPrep PCR purification kit (Stratagene). EMSA was performed as described (Omori and Wall, 1993) with the exception of 5 μg poly(dI·dC)

and 4.5% polyacrylamide/bis-acrylamide 60:1 and 0.5× TBE (22 mM Tris base, 7 mM boric acid, 0.5 mM EDTA pH 8). Gels were run at 125 V for 2.5 h at room temperature. *pp52* NRE EMSA probes were Alu1 DNA restriction fragments (−324 to −235) labeled at both 5′ ends with [γ-^{32}P]ATP. *pp52* anti-NRE probes were the following double-stranded complementary oligonucleotides: 5′-AGCAGTCCACGGACACTAAAGGGACCATGAATT-3′ (*pp52* anti-NRE). *pp52* NRE and *pp52* anti-NRE EMSA probes were purified by G50 and G25 sephadex spin column chromatography, respectively.

3. Results

3.1. pp52 promoter deletion analysis suggests the presence of silencer elements

Previously, we reported that the *pp52* promoter fragment (−549) is tissue-specific and only active in leukocyte cell lines. This *pp52* promoter fragment (−549) had little or no activity in 3T3 fibroblasts (Omori et al., 1997). In order to identify regions of potential tissue-specific activity, we tested two additional deletion constructs in a pre-B cell line (3-1), a B cell line (M12), two T cell lines (BW5147 and EL-4), a macrophage cell line (Raw264), and a fibroblast cell line (NIH 3T3). Table 1 shows that leukocyte-specificity is lost upon deletion to the −99 bp *pp52* promoter. The −99 *pp52* promoter construct was expressed in 3T3 fibroblasts where the −549 *pp52* promoter construct was not. Interestingly, the −350 *pp52* promoter construct was not expressed well in any of the cell lines, suggesting that silencer elements may play a role in *pp52* promoter regulation. To further delineate the putative silencer region and the tissue-specific promoter, a deletion series between −549 and −99 was generated giving rise to *pp52* (−473), *pp52* (−428), *pp52* (−383), *pp52* (−350), *pp52* (−307), *pp52* (−257), *pp52* (−204), and *pp52* (−99). These were tested in the B cell line M12 and the fibroblast cell line 3T3. A substantial and reproducible loss of leukocyte-specific regulation of the *pp52* promoter occurred upon deletion of 5′ sequences −257 bp and smaller to −99 bp (Fig. 1A). These truncated *pp52* promoter fragments (−257, −204, and −99) allowed statistically significant *pp52* promoter activity in 3T3 fibroblasts (Fig. 1A). In contrast, these *pp52* promoter construct activities were unaltered compared to the leukocyte-specific *pp52* (−549) promoter in the M12 B cell line (Fig. 1B). These data also indicate that the *pp52* (−99) fragment is the basal *pp52* promoter.

Table 1
Transcriptional activity of *pp52* gene segments in transient transfection[a]

	Percent minimal *pp52* (−549) promoter[b]					
	Transfected cell lines					
Construct	M12	3-1	BW5147	EL-4	Raw264	NIH 3T3
pCAT basic	6.3	4.0	3.4	2.4	1.2	40.0 (14.3)[c]
pCAT −549	100	100	100	100	100	100 (35.7)[c]
pCAT −350	31.3	26.7	65.0	68.9	3.8	80.0 (28.6)[c]
pCAT −99	93.8	89.0	95.0	n.d.	136.3	280.0 (100)[c]

[a] CAT activities of the *pp52* promoter deletion constructs are expressed as the percentage of the minimal (−549) promoter construct and are the averages of at least three independent transfections. All values were normalized to RSV luciferase activity. M12 is a B cell line, 3-1 is a pre-B cell line, BW5147 is a T cell line, EL-4 is a T cell line, Raw264 is a macrophage cell line, and NIH 3T3 is a fibroblast cell line.

[b] Absolute fold inductions of *pp52* (−549) over pCAT basic for the above cell lines ranged from: 10–21 fold for M12; 14–26 fold for 3-1; 17–40 fold for BW5147; 22–54 fold for EL-4; 29–98 fold for Raw264; and 1–3 fold for NIH 3T3.

[c] CAT activities expressed as the percentage of the (−99) promoter. Absolute fold inductions of *pp52* (−99) over pCAT basic ranged from 4–11 fold.

Interestingly, the deletion constructs *pp52* (−307) and *pp52* (−350) showed significantly decreased activity compared to the *pp52* (−99) in both B cells and fibroblasts, suggesting that there may be tissue-restricting silencer elements within the region between −257 and −383 of the *pp52* promoter (Fig. 1A,B). Fig. 1A,B suggest that the putative *pp52* silencer element(s) were able to down regulate transcription in both B cells and fibroblasts. Fig. 1A,B show that the −383 fragment of the *pp52* promoter was highly active in M12 B cells but not active in 3T3 fibroblasts. These data suggest that there may be sequence elements within the *pp52* (−383) fragment that can counteract the effects of the negative regulatory −257 to −350 region in B cells, but not in fibroblasts (Fig. 1A,B). These studies suggest that the *pp52* promoter contains both silencer (*pp52* NRE) and anti-silencer (designated *pp52* anti-NRE) elements that control its tissue-specific expression (See Fig. 6).

3.2. The pp52 NRE and pp52 anti-NRE forms specific protein complexes in M12 B cell and 3T3 fibroblast nuclear extracts

We tested the *pp52* NRE and the *pp52* anti-NRE elements for their ability to interact with DNA-binding proteins. Fig. 2 shows that DNA-binding proteins from both M12 B cell and 3T3 fibroblast nuclear extracts form a complex with the 89 bp *pp52* NRE. While this complex was specifically competed away by excess unlabeled *pp52* NRE probe, unlabeled oligonucleotides corresponding to the consensus binding site for the YY1 silencer element (Galvin and Shi, 1997) were unable to act as competitors (Fig. 2). In addition, the *pp52* NRE protein complex was not competed away by several known silencer element binding motifs including the ubiquitous B29 gene silencer sites TOAD, FROG, and A + T-rich motif (data not shown). The 33 bp *pp52* anti-NRE also forms specific protein complexes with both M12 B cell and 3T3 fibroblast nuclear extracts (Fig. 3). These complexes are specifically competed away by excess unla-

beled *pp52* anti-NRE oligonucleotides, but are not competed away by unlabeled *pp52* NRE fragment (Fig. 3). These data suggest that the *pp52* NRE and the *pp52* anti-NRE elements interact with differing protein complexes.

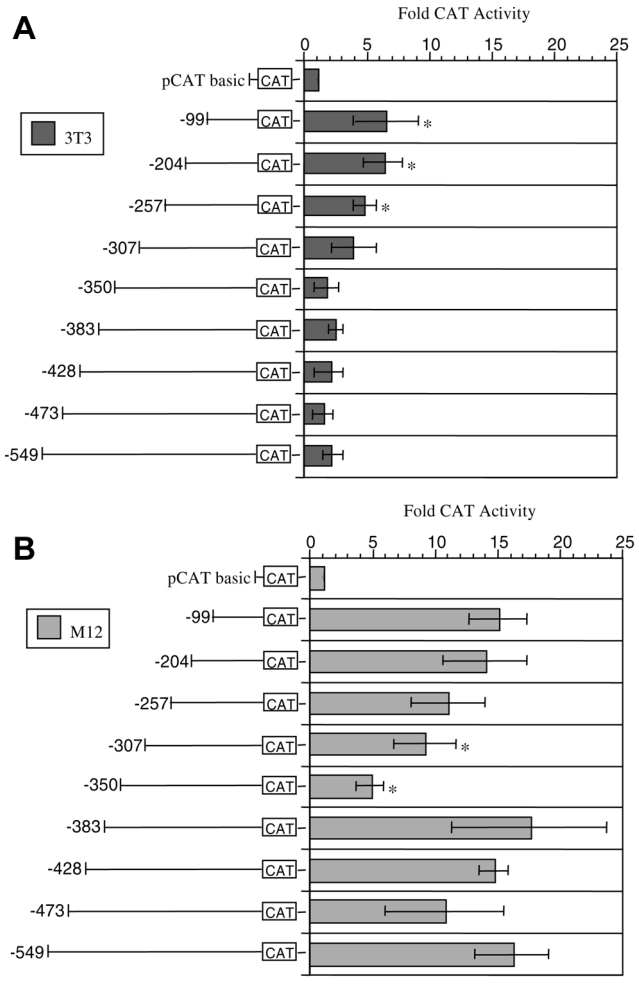

Fig. 1. *pp52* deletion analysis suggests the presence of silencer and anti-silencer elements that control tissue-specificity. Transient transfection to detect transcriptional expression of *pp52* promoter deletion constructs. Deletion constructs are identified by nucleotide number with respect to the start site of transcription (+1). The activity of each construct is expressed as fold induction above pCAT basic promoterless construct. CAT activities are RSV-luciferase normalized and are the averages ±SD of six independent transfections using at least three preparations of DNA. (A) Transient transfections were conducted in the NIH 3T3 fibroblast line (black bars). The Student's two sided *t*-test *P* values for deletion constructs with respect to the tissue-specific *pp52* −549 construct are as follows: −99: $P < 0.05$; −204: $P < 0.05$; −257: $P < 0.05$; −307: $P > 0.05$; −350: $P > 0.05$; −383: $P > 0.05$; −428: $P > 0.05$; −473: $P > 0.05$. Statistically significant values are signified with an asterisk (*). (B) Transient transfections were conducted in the M12 B cell line (gray bars). The Student's two sided *t*-test *P* values for deletion constructs with respect to the basal *pp52* −99 and tissue-specific *pp52* −549 constructs are as follows: −204: $P > 0.05$; −257: $P > 0.05$; −307: $P < 0.05$; −350: $P < 0.05$; −383: $P > 0.05$; −428: $P > 0.05$; and −473: $P > 0.05$. Statistically significant values are signified with an asterisk (*).

3.3. The pp52 NRE acts as a position and orientation independent silencer element

Silencer elements are often inhibitory elements that function in a position and orientation independent manner (Cao et al., 1989; Farrell et al., 1990; Sawada et al., 1994). To determine whether the *pp52* promoter has silencer element activity, an 89 bp Alu1 restriction fragment encompassing most of the transcriptionally inhibited region, −325 to −234 designated the *pp52* NRE, was taken out of context and subcloned immediately 5′ of the *pp52* (−99) minimal promoter construct. As shown in Fig. 4, the *pp52* NRE inhibited the activity of the *pp52* (−99) promoter to the levels of the relatively inactive *pp52* (−350) fragment in both B cells and fibroblasts. Additionally, the *pp52* NRE was able to exert silencing activity in both the forward and reverse orientations (Fig. 4). These data suggest that the *pp52* NRE is a position and orientation independent silencer element. Furthermore, three-fold multimerization of this fragment increased inhibition of the *pp52* (−99) construct in both fibroblasts and B cells, suggesting an addi-

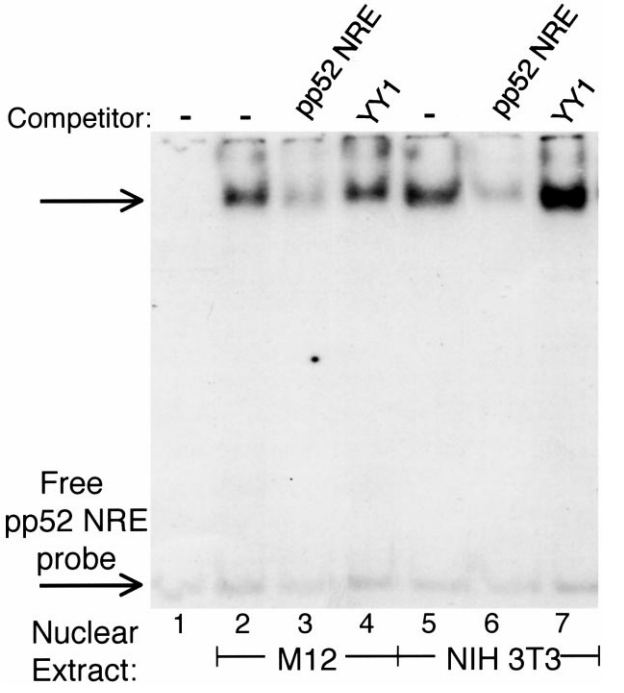

Fig. 2. The *pp52* NRE interacts with DNA-binding proteins. Double-stranded DNA restriction fragment (Alu1) corresponding to the *pp52* promoter sequence −324 to −235 with respect to the transcriptional start site of the *pp52* gene was end-labeled and used in EMSA. Lane 1 contains *pp52* NRE probe alone while lanes 2–4 and 5–7 contain *pp52* NRE probe incubated with 20 μg M12 B cell nuclear extract and 20 μg NIH 3T3 fibroblast nuclear extract, respectively. Probe was also coincubated with 500-fold excess of unlabeled *pp52* NRE probe (lanes 3 and 6) and with 500-fold excess of unlabeled YY1 consensus binding site (lanes 4 and 7). Specifically formed complex is indicated by an arrow and is representative of at least three independent experiments. Unbound probe is indicated by an arrow labeled with 'Free *pp52* NRE probe'.

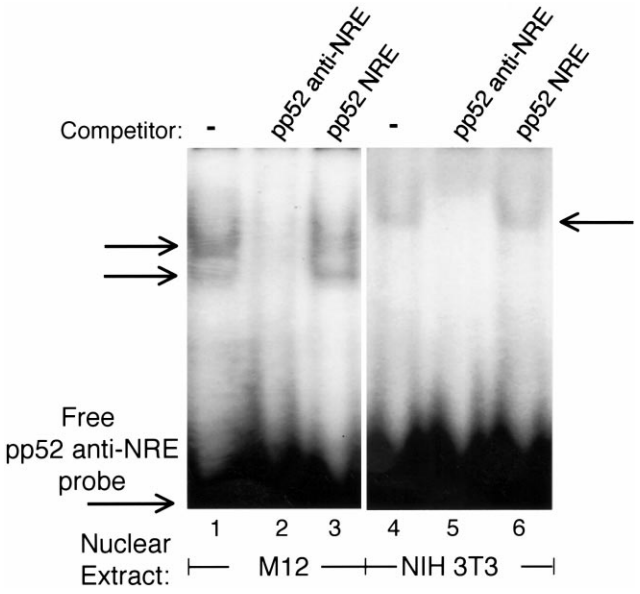

Fig. 3. The *pp52* anti-NRE probe interacts with DNA-binding proteins. Double-stranded oligonucleotides corresponding to the *pp52* promoter sequence −383 to −350 with respect to the transcriptional start site of the *pp52* gene were end-labeled and used in EMSA. Lanes 1–3 contain the *pp52* anti-NRE probe incubated with 20 μg M12 B cell nuclear extract and lanes 4–6 contain the *pp52* anti-NRE probe incubated with 20 μg NIH 3T3 fibroblast nuclear extract. Probe was also coincubated with 500-fold excess of unlabeled *pp52* anti-NRE probe (lanes 2 and 5) and with 500-fold excess of unlabeled *pp52* NRE probe (lanes 3 and 6). Specifically formed complexes are indicated by arrows and are representative of at least three independent experiments. Unbound probe is indicated by an arrow labeled with 'Free *pp52* anti-NRE probe'.

tive effect of multiple elements (Fig. 4). Conversely, when the *pp52* NRE was placed 5′ of the SV40 early promoter (pCATpromoter, Promega), no inhibitory activity was observed (data not shown). Therefore, the *pp52* NRE appears to be an active silencer element in multiple cell types, but it may be specific for *pp52* and not active against heterologous promoters. Several other silencer elements appear to be gene specific as well, including those in the β-*type globin* gene, the *osteocalcin* gene, and the mouse and human *IL-4* gene (Li-Weber et al., 1992; Bruhn et al., 1993; Frenkel et al., 1994; Wandersee et al., 1996).

3.4. Deletion of the pp52 NRE from the pp52 promoter constructs results in alleviation of silencing activity

The *pp52* NRE (−324 to −235) was deleted from the *pp52* (−350), *pp52* (−383), *pp52* (−428), *pp52* (−473), and *pp52* (−549) promoter constructs to clarify the role of the *pp52* NRE in the leukocyte-specific regulation of the *pp52* gene and to determine whether anti-silencer elements were present upstream of the *pp52* NRE. These constructs were tested for transcriptional activity in transient transfection of NIH 3T3 fibroblasts and M12 B cells. Fig. 5A shows that the inhibitory effects of the *pp52* NRE in 3T3 cells were reversed upon the removal of the *pp52* NRE, confirming that the *pp52* NRE sequence was responsible for the silencing of the *pp52* promoter constructs in fibroblasts. These data therefore suggest that the *pp52* NRE controls the leukocyte-specific activity of the *pp52* promoter. Fig. 5B shows that the inhibitory effects of the *pp52* NRE in B cells were reversed upon removal of these sequences in the *pp52* (−350-NRE) promoter, confirming the observation from Fig. 1 that the *pp52* NRE functions as a silencer element in B cells as well as in fibroblasts. Removal of the *pp52* NRE in the other *pp52* promoter constructs (−383-NRE, −428-NRE, −473-NRE, and −549-NRE) in B cell transfections showed no significant difference from the comparable wild type promoter construct (−383, −428, −473, −549) (Fig. 5B). These data are consistent with the observation from Fig. 1 that the *pp52* NRE exerts no silencing activity in the *pp52* (−383), *pp52* (−428), *pp52* (−473), and *pp52* (−549) promoter constructs in B cells. Additionally, no significant difference in promoter activity between the wild type and the NRE deletion mutant constructs (e.g. −383 versus −383-NRE) was observed, suggesting that the *pp52* anti-NRE has no inherent enhancer activity, and can therefore be defined as an anti-silencer element. Together, these results suggest that the *pp52* anti-NRE anti-silencer element was active in B cells, but not in fibroblasts. Collectively, these data suggest that the −324 to −235 *pp52* NRE

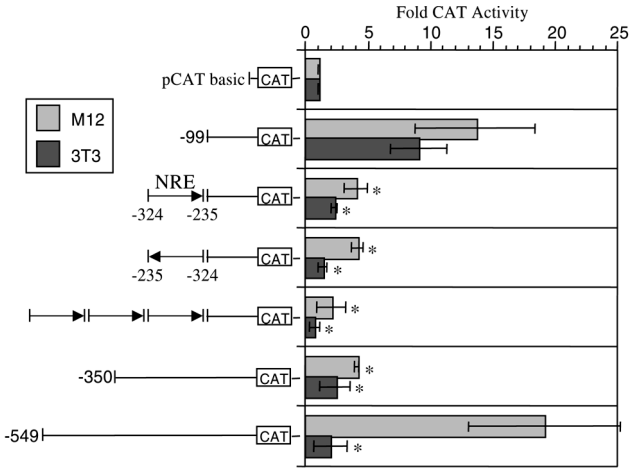

Fig. 4. The *pp52* NRE acts as a position and orientation independent silencer element. Transient transfections to detect transcriptional expression of *pp52* promoter/NRE constructs were conducted in the M12 B cell line (gray bars) and NIH 3T3 fibroblast line (black bars). The *pp52* NRE fragments and the position and orientation relative to the *pp52* (−99) promoter are as indicated. Deletion constructs −99, −350, and −549 are identified by nucleotide number with respect to the start site of transcription (+1) and are shown for comparison. The activity of each construct is expressed as fold induction above pCAT basic promoterless construct. CAT activities are RSV-luciferase normalized and are the averages ±SD of six independent transfections using at least three preparations of DNA. The Student's two sided *t*-test *P* values for deletion constructs with respect to the minimal *pp52* −99 construct are as follows: single NRE forward, M12: $P < 0.05$, 3T3: $P < 0.05$; single NRE reverse, M12: $P < 0.05$, 3T3: $P < 0.05$; triple NRE forward, M12: $P < 0.05$, 3T3: $P < 0.05$; −350, M12: $P < 0.05$, 3T3: $P < 0.05$; and −549, M12: $P > 0.05$, 3T3: $P < 0.05$. Statistically significant values are signified with an asterisk (*).

silencer element is solely responsible for shutting off *pp52* expression in fibroblasts while the *pp52* anti-NRE anti-silencer element between −350 and −383 overcomes the *pp52* NRE silencer element activity in B cells and allows promoter activity.

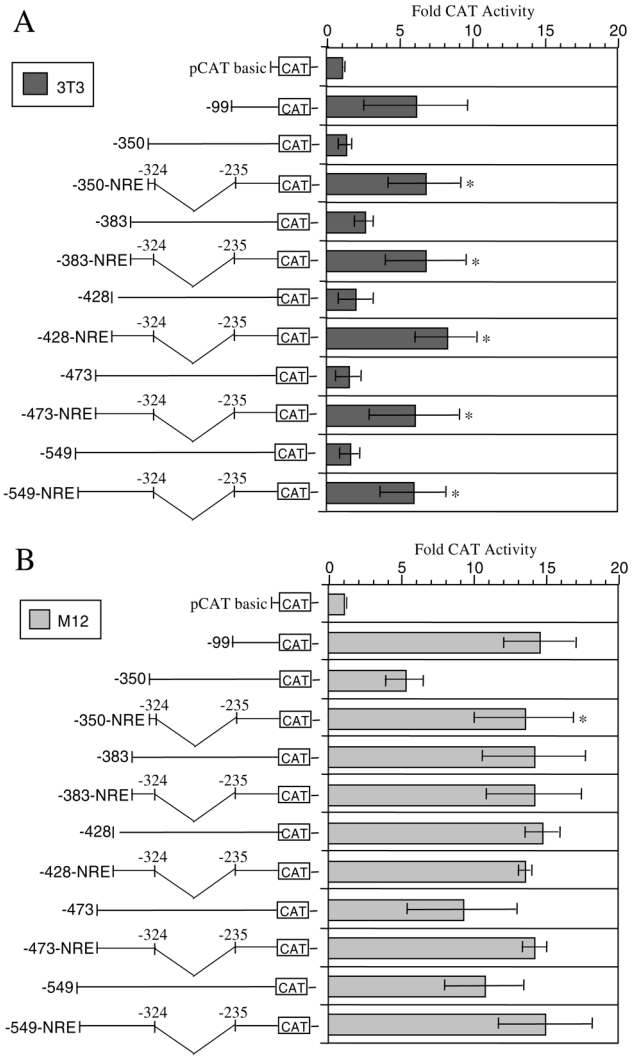

Fig. 5. Deletion of the *pp52* promoter constructs results in alleviation of silencing activity. Transient transfections to detect transcriptional expression of *pp52* promoter/NRE deletion constructs were conducted in the (A) NIH 3T3 fibroblast line (black bars) and (B) M12 B cell line (gray bars). Constructs with the deletion of the *pp52* NRE are indicated by having a gap in the promoter construct and by being labeled with '-NRE', e.g. '-350-NRE'. Deletion constructs −99, −350, −383, −428, −473, and −549 are identified by nucleotide number with respect to the start site of transcription (+1) and are shown for comparison. The activity of each construct is expressed as fold induction above pCAT basic promoterless construct. CAT activities are RSV-Iuciferase normalized and are the averages ±SD of six independent transfections using at least three preparations of DNA. The Student's two sided *t*-test *P* values for deletion constructs with respect to their corresponding promoter construct containing the NRE are as follows: −350-NRE, M12: $P < 0.05$, 3T3: $P < 0.05$; −383-NRE, M12: $P > 0.05$, 3T3: $P < 0.05$; −428-NRE, M12: $P > 0.05$, 3T3: $P < 0.05$; −473-NRE, M12: $P > 0.05$, 3T3: $P < 0.05$; and −549-NRE, M12: $P > 0.05$, 3T3: $P < 0.05$. Statistically significant values are signified with an asterisk (*).

4. Discussion

Our studies delineate a silencer/anti-silencer cassette located within the 549 bp leukocyte-specific *pp52* promoter that restricts basal promoter (−99 to +1) activity in non-leukocyte cells (see Fig. 6). We have narrowed the *pp52* silencer region to an 89 bp fragment (*pp52* NRE, −324 to −235) and have shown that this fragment alone, out of context and in either orientation, was sufficient to inhibit the activity of the *pp52* (−99) promoter in fibroblasts and B cells. The activity of this *pp52* silencer element in B cells, where *pp52* is normally expressed, places the *pp52* NRE into an emerging class of transcription silencer elements which are also active within their own cell lineages. This silencer family includes *B29* (Malone et al., 1997), *bcl-2* (Young and Korsmeyer, 1993), hu*ETS-1* (Chen et al., 1993), and λ*5* (Martensson and Melchers, 1994; Yang et al., 1995). Interestingly, all of these known silencers control genes that have TATA-less promoters (Dynan and Tjian, 1983; Oka et al., 1991; Omori and Wall, 1993; Martensson and Melchers, 1994). Unlike these other silencers, the *pp52* silencer activity appears to be overcome by 5′ sequences in B cells where *pp52* is expressed, but not in fibroblasts where *pp52* is not normally expressed. Such results are indicative of anti-silencer activity conferring tissue-specific transcriptional control (Stover and Zehner, 1992; Ferradini et al., 1994; Dobretsova and Wight, 1999; Izmailova and Zehner, 1999). This anti-silencer was located between −350 and −383 in the *pp52* promoter (*pp52* anti-NRE) and allowed activity of the −383 and larger *pp52* promoter constructs in B cells. This anti-NRE was not active in fibroblasts, and therefore did not derepress the *pp52* promoter construct containing the *pp52* NRE (e.g. −383, −472, −549). The *pp52* promoter was only expressed in fibroblasts with constructs lacking the *pp52* NRE such as the −207 and −99 promoter constructs, or when the *pp52* NRE (−324 to −235) was deleted from the −383 and larger *pp52* promoter constructs. These data suggest the *pp52* NRE is responsible for the inhibition of *pp52* expression in fibroblasts. In B cells, the *pp52* (−549) promoter is highly active regardless of the presence of the *pp52* NRE, when the *pp52* anti-NRE is present. Anti-silencer elements have the ability to over-ride transcriptional inhibition by silencer elements, but have no innate enhancer activity on their own (Stover and Zehner, 1992; Ferradini et al., 1994; Dobretsova and Wight, 1999; Izmailova and Zehner, 1999). The *pp52* promoter clearly fits this anti-silencer criteria as it over-rides the *pp52* NRE, but it does not substantially increase or enhance the activity of the *pp52* basal promoter.

Several functional silencer elements and anti-silencer elements are reported to interact with DNA binding proteins (Stover and Zehner, 1992; Ferradini et al., 1994; Ernst and Smale, 1995; Dobretsova and Wight, 1999; Izmailova and Zehner, 1999). DNase 1 protection analyses over a large portion of the *pp52* promoter failed to delineate a limited region of DNA binding activity, and instead predicted a

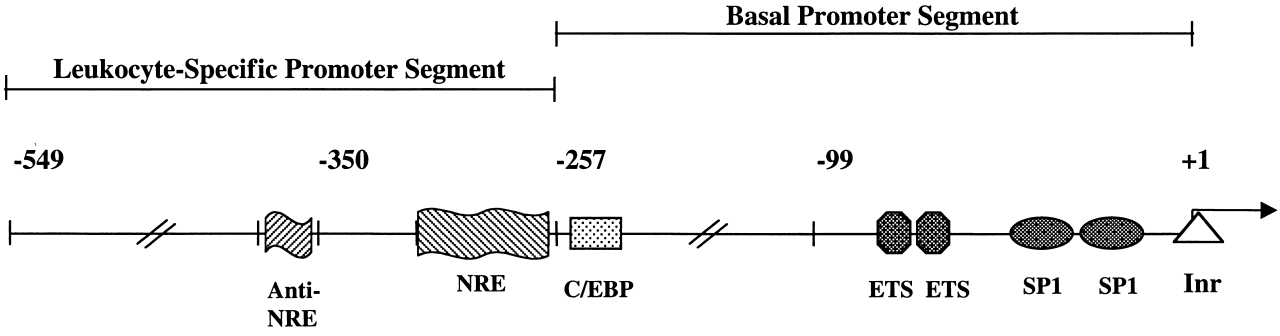

Fig. 6. Schematic of the regulatory regions and transcription factors identified within the *pp52* promoter. The *pp52* −549 tissue-specific promoter was identified and characterized to be controlled by C/EBP, Ets, and Sp1 factors (Omori et al., 1997). Deletion analyses identified the *pp52* −99 basal promoter, silencer (NRE) and anti-silencer (anti-NRE) segments. Anti-NRE and NRE elements are shown as they reside in the *pp52* promoter relative to sequence number and to position of known transcription factor binding sites.

large protein complex spanning both the *pp52* NRE and the *pp52* anti-NRE (data not shown). In our EMSA analyses, the *pp52* NRE fragment formed specific protein complexes in both M12 and 3T3 nuclear extracts in EMSA that appeared to have the same mobility and similar affinity between the two cell types. These nuclear protein binding results support the functional data showing that the *pp52* NRE is active in both B cells and fibroblasts. The identity of the protein(s) interacting with the *pp52* NRE remains to be determined. EMSA using the defined 33 bp *pp52* anti-NRE resulted in a differing pattern of specific protein complex formation in M12 and 3T3 nuclear extracts. These data suggest that the *pp52* anti-NRE interacts with different proteins or protein complexes between the two cell types. These results support our functional data showing that the *pp52* anti-NRE had differing activities in M12 B cells and 3T3 fibroblasts, possibly due to the differing protein complexes seen in EMSA.

The transient transfection functional data clearly demonstrated a role for both the *pp52* NRE silencer and the *pp52* anti-NRE anti-silencer in controlling *pp52* gene expression. Similarly acting silencer and anti-silencer elements have been identified within both the human and chicken intermediate filament *vimentin* gene promoters (Stover and Zehner, 1992; Izmailova and Zehner, 1999), the chicken *lambda light chain* locus (Ferradini et al., 1994), the *myelin proteolipid protein* gene (Dobretsova and Wight, 1999), and the Escherichia coli gene *RcsA* (Sledjeski and Gottesman, 1995). Like the *pp52* anti-silencer, the human and chicken *vimentin* anti-silencer elements were shown to restore *vimentin* gene activity in cell types where the *vimentin* gene is expressed. Reminiscent of the fact that *pp52* anti-silencers do not function as enhancer elements, the *vimentin* gene anti-silencers also do not affect gene activity when their corresponding silencer element is absent (Stover and Zehner, 1992; Izmailova and Zehner, 1999). Gene regulation of the *lambda light chain* is even more comparable to the mechanism we found for *pp52* gene regulation. The tissue-specific *lambda light chain* gene rearrangement and subsequent transcription is controlled by a silencer/anti-silencer interplay where the silencer element is active in both orientations in all cell types and the anti-silencer is only active in B cells (Ferradini et al., 1994). The *lambda light chain* gene data suggest that during B cell development, only B cell precursors express the anti-silencer binding proteins that counteract the effect of the silencer, thereby allowing rearrangements and *light chain* gene transcription to occur. In the case of the *pp52* gene, expression of factors that bind to the anti-NRE is presumably restricted to leukocytes. The interactions of such factors with the anti-NRE would over-ride the otherwise dominant inhibitory effects of the *pp52* NRE silencer, thereby allowing *pp52* gene expression only in leukocytes. These findings add the *pp52* gene to a growing list of genes in which anti-silencers control tissue-specific gene transcription by relieving normal gene repression mediated by non-tissue restricted silencers. Interestingly, this complex mode of gene regulation appears to be the rule rather than the exception for genes encoding proteins of highly specialized functions that are restricted to specific cell lineages.

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