Characterization of the human proline/arginine-rich end leucine-rich repeat protein (PRELP) gene promoter and identification of a repressor element

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The 5′-flanking region of the human proline/arginine-rich end leucine-rich repeat protein (PRELP) gene has been characterized for both promoter and repressor activity by using a variety of reporter gene constructs and transient transfection into chondrocytes or fibroblasts. The human PRELP gene lacks a TATA box, and in its absence a Sp1-binding site residing 29 bp upstream of the transcription start site is essential for initiating gene expression. In contrast, an Ets-binding site residing 497 bp upstream of the transcription start site can lead to the repression of gene expression. The analysis of nuclear proteins by gel retardation studies with the repressor element identified a common protein, presumably an Ets family member, present in neonatal chondrocytes and skin fibroblasts that do not express the PRELP gene. The factor was not detected in nuclear protein preparations from adult chondrocytes in which the PRELP gene is expressed.

INTRODUCTION

PRELP (proline/arginine-rich end leucine-rich repeat protein) belongs to the large family of leucine-rich repeat (LRR) proteins [1,2]. More specifically, it is a member of the subfamily that contains a variety of small LRR proteoglycans (SLRPs), including the dermatan sulphate-proteoglycans decorin and biglycan, and the keratan sulphate-proteoglycans fibromodulin and lumican [3]. This subfamily is characterized by the presence of 10 adjacent LRRs, flanked at either end by disulphide-bonded domains. N-linked oligosaccharides are present within the central LRR domain, and in fibromodulin and lumican these oligosaccharides form the point of attachment for keratan sulphate. Under some circumstances it seems that PRELP might also be substituted with keratan sulphate in this region [1], though it is more commonly present as a glycoprotein devoid of keratan sulphate [4–6]. The subfamily members show the greatest degree of structural variation in their N-terminal domains, which in the case of decorin and biglycan contain the sites of attachment for dermatan sulphate. For PRELP it is the high abundance of proline and arginine residues in this region that led to its name.

In the human, the PRELP gene resides on chromosome 1q32 and is encoded by three exons [6]. The gene gives rise to messages of 1.7, 4.6 and 6.7 kb owing to the use of different polyadenylation signals, with the smallest message being most abundant. The messages possess a single transcription start site residing 199 nucleotides before the translation initiation codon. The proximal promoter region preceding this site contains numerous potential transcription factor-binding sites, but contains no CAAT or TATA boxes to locate the transcription machinery. In human articular cartilage it is apparent that expression of the PRELP gene is not constant throughout life, with message levels being deficient in the foetus and newborn [6]. In accord with this lack of message expression, there is little evidence for PRELP's being present in the extracellular matrix of foetal or neonatal cartilage [5,6]. This is in contrast with the abundant production of both PRELP message and protein later in life.

The purpose of the present study was to identify the promoter element responsible for the transcription of the PRELP gene in the absence of a TATA box, to identify the repressor element within the promoter responsible for down-regulation of PRELP gene expression in the newborn, and to identify the transcription factor family responsible for that down-regulation.

METHODS

Promoter sequence

The sequence of the promoter region was determined by direct sequencing of a previously identified human cosmid clone [6], using the Cyclist Exo$^-$Pfu DNA sequencing kit (Stratagene, La Jolla, CA, U.S.A.) commencing with primers from the published cDNA sequence [6] and then using subsequent primers generated from the extended sequence.

Reporter gene constructs

The pGL2 series of luciferase reporter gene plasmids (Promega, through Fisher, Nepean, ON, Canada) was used for all PRELP promoter/reporter gene constructs. The required DNA fragments from the PRELP promoter region were amplified by PCR from human genomic DNA by using standard protocols [7] and primers chosen from the sequence generated as described above. Amplified fragments were cloned into the pCR2.1 plasmid with the TOPO TA cloning kit (Invitrogen, San Diego, CA, U.S.A.), in accordance with the manufacturer's instructions. Orientation of the cloned fragment was determined by sequence analysis with the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham, Oakville, ON, Canada). Depending on orientation, the endonucleases *Sac*I plus *Xho*I, or *Hin*dIII plus *Xho*I, were used to excise the fragment from the pCR2.1 plasmid for ligation into the luciferase reporter gene pGL2B plasmid in the desired orientation. Various promoter 5′-deletion fragments were used in reporter gene constructs to identify transcriptionally active elements: A (bp −31 to 40), B (bp −38 to 40), C (bp −125 to 40), D (bp −292 to 40), E (bp −564 to 40), F (bp −870 to 40)

Abbreviations used: EBS, Ets-binding site; LRR, leucine-rich repeat; PRELP, proline/arginine-rich end leucine-rich repeat protein.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U41292.

and fragment EBS (Ets-binding site) (bp −509 to 40). In addition, the PRELP promoter repressor element region (bp −564 to −123) was cloned into the pGL2P plasmid containing the SV40 promoter, by the same methods. All numbering refers to Figure 1. Synthetic oligonucleotides, 5′-CTCCGAACCTC-CCTCCCTATGAT-3′ and 5′-CCAAACCGCTAGTTATCTC-CT-3′, were used as upstream primers to introduce mutations into the Sp1 site (fragment B_m) and the EBS (EBS_m) respectively. The downstream primer in each mutagenesis amplification was 5′-CTTCCACCAGATCCTATT-3′. The amplified fragments containing the mutated sequences were cloned into the luciferase reporter gene pGL2B plasmid, as above.

Source of human tissue and cells

Human articular cartilage was collected from the distal femur at the time of autopsy and within 20 h of death. The specimens were from individuals aged 2 months and 33, 48, 63 and 67 years. In all cases the knee joints were of macroscopically normal appearance and there was no clinical evidence of a connective tissue abnormality. Chondrocytes were isolated and grown in monolayer culture, as described previously [6]. Fibroblasts were isolated from full-thickness skin biopsies obtained from the forearm of individuals aged 41 and 43 years, and grown in monolayer culture by the method previously described [8].

Cell transfections

Only chondrocytes with three passages or less in culture were used for transfection. Adult chondrocytes or fibroblasts were plated at a density of 10^5 or 5×10^4 cells respectively in each 60 mm tissue culture dish 3 days before transfection. Transfections were performed with lipid reagent Pfx-6 from the Perfect Transfection Kit (Invitrogen, San Diego, CA, U.S.A.), in accordance with the manufacturer's instructions. Cells were co-transfected for 4 h with a mixture of 4 μg each of experimental and control reporter gene constructs, with a lipid-to-DNA ratio of 6:1. The pRL-TK plasmid (Promega, through Fisher) was used as an internal control in all transfections. All transfections were repeated in triplicate.

Luciferase assay

The dual-luciferase reporter assay system (Promega, through Fisher) was used as described by the manufacturer, except that assays were performed 72 h after transfection. Luciferase activity was measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, U.S.A.). Control constructs utilized the *Renilla* luciferase and experimental constructs utilized the firefly luciferase. Untransfected cells were used to generate background levels of light measurement. The activity of each construct was calculated from the expression [(experimental luciferase activity) − background]/[(control luciferase activity) − background]. Results are reported as the means ± S.D. for three separate experiments.

Gel retardation assay

Nuclear extracts were prepared from neonatal and adult chondrocytes [9]. Synthetic double-stranded oligonucleotide probes spanning bp −564 to −507 (probe A), bp −526 to −452 (probe B) and bp −466 to −407 (probe C) were prepared, leaving a 6 bp overhang at the 5′ end of each strand. Probes were radiolabelled with [^{32}P]dCTP by using a standard fill-in reaction [7] with the small fragment of DNA polymerase I (Klenow), and purified on a 5% (w/v) non-denaturing polyacrylamide gel. Nuclear extract (5 μg) was incubated with 50000 c.p.m. of radiolabelled probe, with or without a competitor, in a 20 μl reaction containing 12 mM Hepes/NaOH, pH 7.9, 4 mM Tris/HCl, pH 7.9, 60 mM KCl, 1 mM dithiothreitol, 6% (v/v) glycerol and 5 μg of poly(dI-dC)·poly(dI-dC). After 30 min of incubation at room temperature, reactions were analysed on a 5% (w/v) non-denaturing polyacrylamide gel containing 89 mM Tris/HCl, pH 8.0, 89 mM boric acid and 2 mM EDTA. After electrophoresis, gels were dried and autoradiographed. In addition to non-labelled probes, the following synthetic double-stranded oligonucleotides were used as competitors to radiolabelled probes: bp −526 to −483 (competitor B1), bp −489 to −461 (competitor B2), and bp −512 to −491 (EBS).

RESULTS

A human genomic DNA clone was used to provide nucleotide sequence data for the PRELP gene promoter. A continuous sequence of 1076 bp was obtained (Figure 1) immediately upstream of the published transcription start site [6]. Computer analysis of the promoter sequence revealed the presence of numerous potential binding sites for vertebrate-encoded transcription factors [10] on both DNA strands (Figure 1). Of particular interest was the presence of an Sp1-binding site lying 29 bp upstream of the transcription start site. This is of interest because of the absence of CAAT and TATA elements from the proximal promoter region, and Sp1 binding has been described as an alternative means of locating the transcription machinery [11]. Therefore this element is a candidate for allowing the basal expression of the PRELP gene. This Sp1 site forms part of a prominent CT element, which has also been associated with the initiation of transcription [12]. In addition, the more distal promoter contains several EBSs. These are of particular interest because members of this family have previously been associated with the down-regulation of gene transcription [13] and therefore could be candidates for facilitating the repression of the PRELP gene in both neonatal cartilage and non-cartilagenous tissues. The promoter region is also of note for possessing two nucleotide sequences (CIIS1 and CIIS2) that have previously been reported

Figure 1 DNA sequence of the promoter region of the PRELP gene

The promoter region is numbered from −1076 to −1 and the transcribed sequence is numbered from 1 to 70. Potential transcription factor-binding sites are indicated above the sequence, and the two sites of particular interest to the present study are underlined.

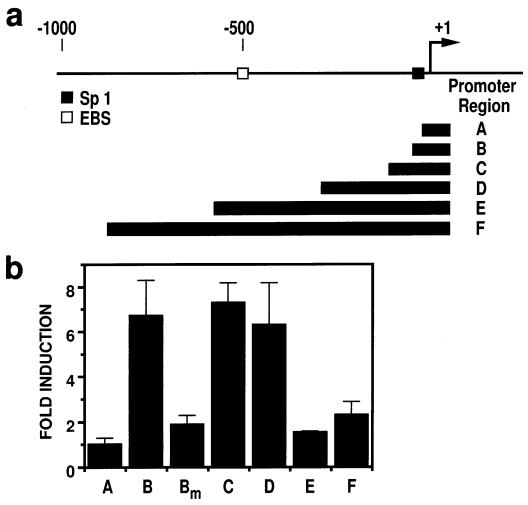

Figure 2 Luciferase reporter gene analysis of the PRELP promoter

(**a**) Schematic representation of the PRELP promoter, indicating the location of promoter regions (A, B, C, D, E and F) used for generating promoter/luciferase constructs for transient transfections in adult human chondrocytes. The location of the important Sp1-binding site and EBS are shown. (**b**) Luciferase activity in the PRELP promoter constructs is expressed as fold induction compared with the activity of the control vector. Activity is shown for the regions (A, B, C, D, E and F) depicted in (**a**) as well as for a construct containing a mutation in the Sp1-binding site (B_m).

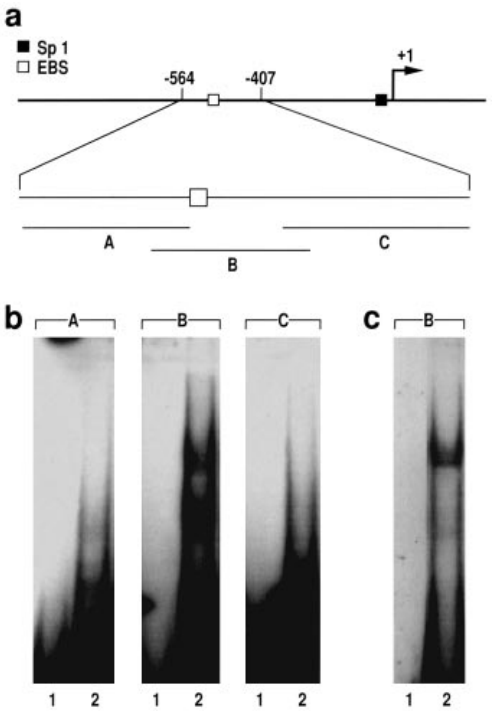

Figure 3 Gel retardation analysis of chondrocyte nuclear proteins

(**a**) Schematic representation of the PRELP promoter indicating the location of the probes (A, B and C) used in the gel retardation assay. The location of the important Sp1-binding site and EBS are shown. (**b**) The electrophoretic mobilities of labelled probes A, B and C are shown in the absence (lanes 1) or presence (lanes 2) of nuclear protein extracted from neonatal chondrocytes. (**c**) The electrophoretic mobility of labelled probe B is shown in the absence (lane 1) or presence (lane 2) of nuclear protein extracted from adult chondrocytes.

as silencers involved in the regulation of the type II collagen gene [14]. As PRELP exhibits a similar tissue specificity in its expression to type II collagen, these elements could also be active in the PRELP promoter.

Initial studies on the PRELP promoter were concerned with identifying the region of the proximal promoter responsible for basal transcription. To achieve this goal, various promoter/luciferase reporter gene constructs were generated that differed only in the site of their upstream terminus (Figure 2a), and transfected into adult human chondrocytes, where PRELP gene expression is known to occur. Promoter activity was then monitored by the analysis of luciferase expression (Figure 2b). The shortest promoter construct encompassing 31 bp upstream and 40 bp downstream of the transcription start site (region A) exhibited no promoter activity. In contrast, when the promoter sequence was extended upstream by 7 bp to include the Sp1 site (region B), a 6-fold induction in promoter activity was observed. Further extension of the promoter sequence to include the entire CT element (region C) or an additional 196 bp (region D) yielded no further variation in promoter activity, suggesting the absence of additional enhancer elements or of repressor elements from the proximal promoter region. The involvement of the Sp1 site in the expression of promoter activity was confirmed by the use of a construct in which the site had been selectively mutated (region B_m). This construct exhibited no promoter activity (Figure 2b).

The promoter/luciferase reporter gene constructs were then extended to encompass the more distal regions of the PRELP promoter (Figure 2a), to determine whether repressor elements might be present. Again, passaged adult chondrocytes were used for transfection. At first sight this might seem an unsuitable cell type for repressor analysis, as PRELP gene expression is known to be highest in adult cartilage [6]. However, PRELP gene expression decreases rapidly on passage in monolayer culture, so it was reasoned that such cells from early passage would be useful for the analysis of both the initiation and repression of PRELP gene expression. This logic seemed valid as both of the extended promoter constructs (regions E and F) showed diminished promoter activity in comparison with the more proximal constructs (Figure 2b). Thus a repressor element seems to reside within the promoter region bounded by nucleotides -564 to -292. This region is of particular interest because it contains two EBSs and the type II collagen silencer elements.

To define more precisely the location of the repressor element, gel retardation assays were performed with three overlapping DNA probes spanning the promoter region containing the potential repressor element(s) (Figure 3a) in combination with nuclear proteins isolated from neonatal chondrocytes (Figure 3b). Neonatal chondrocytes were chosen for this task because they normally show no expression of the PRELP gene. Although low levels of protein interaction were evident with the two flanking probes (A and C), by far the strongest interactions occurred with the central probe (B). In this case two predominantly retarded components were observed. The central region is of note as it contains one of the EBSs. The flanking regions contain the sites for the type II collagen silencer elements and the second EBS, which would therefore seem to be less likely as sequences responsible for repression of the PRELP gene.

If the gel retardation pattern observed with the neonatal nuclear proteins is associated with repression of the PRELP gene, one would expect to see a different electrophoretic pattern if nuclear proteins from adult chondrocytes were used for a similar analysis, because such cells express the PRELP gene. An

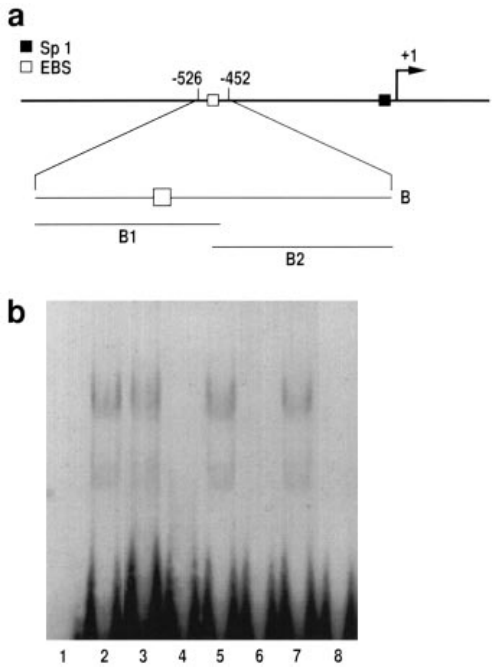

Figure 4 Gel retardation analysis of the PRELP repressor element

(**a**) Schematic representation of the PRELP promoter indicating the location of the labelled probe (B) and probe fragments (B1 and B2) used in binding competition studies. (**b**) The electrophoretic mobility of labelled probe B (lanes 1 to 8) is shown in the absence (lane 1) or presence (lanes 2–8) of nuclear protein extracted from neonatal chondrocytes. Probe plus nuclear extract were incubated without (lane 2) or with a 100-fold excess of unlabelled binding competitor A (lane 3), B (lane 4), C (lane 5), B1 (lane 6), B2 (lane 7), or EBS synthetic oligonucleotide (lane 8). The locations of competitors A and C are as indicated for probes A and C in (**a**).

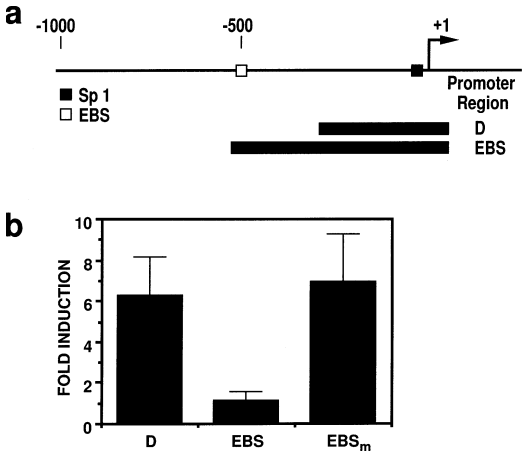

Figure 5 Luciferase reporter analysis of the EBS

(**a**) Schematic representation of the PRELP promoter, indicating the location of promoter regions (D and EBS) used for generating promoter/luciferase constructs for transient transfections in adult human chondrocytes. (**b**) The proximal region of the PRELP promoter (region D) and the more distal region terminating at the EBS were cloned into the luciferase reporter gene vector. A third construct containing the more distal region but with a mutation in the EBS (EBS$_m$) was also analysed. Luciferase activity is expressed as fold induction relative to the activity of the control vector.

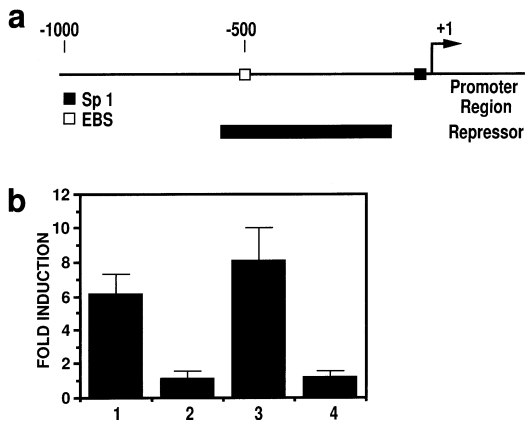

Figure 6 Luciferase reporter gene analysis of the PRELP repressor element

(**a**) Schematic representation of the PRELP promoter, indicating the location of the repressor element region used for generating promoter/luciferase constructs for transient transfections in adult human chondrocytes. (**b**) The repressor element region was cloned into a luciferase reporter gene vector containing the SV40 promoter, and constructs were transfected into fibroblasts (columns 1 and 2) or chondrocytes (columns 3 and 4). Luciferase activity is compared between constructs without the PRELP repressor (columns 1 and 3) and those containing the PRELP repressor (columns 2 and 4), and is expressed as fold induction relative to the activity of the control vector.

altered gel retardation pattern was indeed observed between the nuclear protein preparations (Figure 3c), with the adult preparations being deficient in the more mobile of the retarded components present in the neonatal preparations. The protein responsible for this interaction would therefore seem to be a candidate for causing the repression of PRELP gene expression. This view was supported by the observations that the abundance of a similar component was increased in nuclear protein preparations isolated from adult chondrocytes maintained in monolayer culture, in which PRELP gene expression decreases, and from skin fibroblasts, from which it is absent (results not shown).

To define more fully the specificity of the interactions observed in the gel retardation assay, various DNA sequences were used as competitors for blocking the interaction between probe B and the neonatal chondrocyte nuclear proteins (Figure 4). As expected, the use of unlabelled probe B itself in such analyses competed completely with the interaction of the labelled probe, whereas competitors equivalent to the flanking probes A and C had no effect (Figure 4b, lanes 3 to 5). To define more closely the location of the interacting DNA element, the probe B region was divided into two subregions (B1 and B2; Figure 4a). When these sequences were used as competitors, only the B1 sequence containing the EBS was able to compete with the interaction (Figure 4b, lanes 6 and 7). The involvement of the EBS itself in the interaction was confirmed by performing competition studies in the presence of a synthetic oligonucleotide spanning the EBS. This competed completely with the interaction of the nuclear proteins with the region containing the repressor element of the PRELP gene (Figure 4b, lane 8). This strongly suggests that an Ets family member is responsible for the repression of PRELP gene expression.

To confirm the involvement of the EBS in the repression of PRELP gene expression, promoter/luciferase reporter gene assays were performed (Figure 5) in which the activity of the promoter sequence terminating just upstream of the EBS was

compared with the activity of a proximal promoter sequence terminating downstream of this site (region D). As expected, the presence of the EBS abolished promoter activity. Verification that the EBS was solely responsible for this effect was obtained by use of a construct in which the EBS was selectively mutated (EBS_m). In this case luciferase activity was again generated by the construct. Therefore there seems little doubt that the binding of an Ets protein to an EBS located approx. 500 bp upstream of the transcription start site is responsible for the repression of PRELP gene expression.

To check whether the PRELP gene repressor element could act in a similar manner in other promoters, the region containing the repressor element (bp -564 to -123) was cloned into a luciferase reporter vector containing the SV40 promoter. The construct containing the SV40 promoter alone was active when transfected into either skin fibroblasts or cultured adult chondrocytes (Figure 6, columns 1 and 3), whereas the construct containing the added upstream repressor element was inactive in both cell types (Figure 6, columns 2 and 4). Therefore it seems that the action of the repressor element is not specific for the PRELP gene and that it might be responsible for the repression of gene transcription in different genes and different cell types.

DISCUSSION

The promoter region of eukaryotic protein-encoding genes, encompassing approx. 50 nt adjacent to the transcription start site, commonly possesses a TATA box and/or an initiator element, although some genes possess neither [15]. These elements are involved with locating the transcription machinery, including a variety of general transcription factors and RNA polymerase II. When the TATA box and initiator elements are present, assembly of the transcription machinery commences with the interaction of a TATA-binding protein or an initiator-binding protein. Promoters lacking both TATA box and initiator elements require an additional mechanism for locating the transcription machinery; such promoters commonly have multiple transcription start sites, suggesting that the mechanism is imprecise.

Unlike the TATA box, the initiator element spans the transcription start site [16]. Its structure is imprecise but commonly involves A and T nucleotides at positions $+1$ and $+3$ respectively, surrounded by pyrimidines. In this respect the PRELP promoter is unlikely to possess a strong initiator element, which would be in agreement with the absence of transcription activity in the promoter/luciferase reporter gene construct that spans only the transcription start site (region A; Figure 2a). However, the presence of a weak initiator element cannot be completely discounted. Furthermore initiator elements often act in conjunction with an upstream Sp1-binding site, so the PRELP promoter would be compatible with such a scenario. It is also possible that the Sp1-binding site is solely responsible for locating the transcription machinery [11]. Irrespective of whether it acts in isolation or in conjunction with other elements, it is clear that the presence of a functional Sp1-binding site just upstream of the transcription start site is essential for the expression of the PRELP gene.

It is also clear from the present work that an EBS located approx. 500 nt upstream of the transcription start site is involved in the repression of PRELP gene expression in a variety of situations. This is one of three such sites located within the promoter region examined, but seems to be the only one exhibiting such an effect. The Ets family of transcription factors encompasses at least 12 gene products, including Ets-1, Ets-2, Elk-1 and SAP-1, which might act in isolation or in association with other transcription factors [13]. Ets-1 and Ets-2 have been described as acting in co-operation with the AP1 transcription factor, and Elk-1 and SAP-1 can operate in association with the serum response factor. In this respect the PRELP promoter possesses neither AP1-binding sites nor serum response factor-binding sites within 500 bp upstream or downstream of the repressor EBS. However, such sites might be present further upstream of the promoter region analysed or might not be needed for the observed repressor activity. At present it is not possible to come to definite conclusions on the identity of the Ets family member responsible for repression of PRELP gene expression.

It is interesting to note that there seem to be two nuclear proteins that interact with the repressor EBS. One of these was present in all cell types examined, whereas the other was predominant only in those cells in which PRELP gene expression is deplete. One explanation for this observation is that the EBS could interact constitutively with an Ets family member in all cell types but that in those cells in which PRELP expression is down-regulated a second Ets family member is produced that displaces the constitutive interaction and facilitates repression. It is known that different Ets proteins might compete for occupancy of a common binding site [17]. Further studies will, however, be necessary to prove whether such a situation actually occurs.

At present, characterization of the promoters of the genes giving to rise to the other members of the LRR-protein subfamily to which PRELP belongs is not detailed, but there is sufficient information to allow some comparisons with the PRELP gene promoter to be made. The human and murine biglycan promoters, in common with the PRELP promoter, do not possess CAAT or TATA boxes [18,19]. They do, however, possess numerous GC-rich regions containing several Sp1-binding sites. The human biglycan promoter is similar to the PRELP promoter in that the proximal promoter region containing the first Sp1-binding site is sufficient for generating promoter activity [20]. In the mouse biglycan promoter more distal elements are also required [19]. The human decorin gene promoter possesses two transcription start sites that give rise to different exons (1a and 1b) encoding the upstream part of the 5′-untranslated region of the decorin message [21]. The promoter region giving rise to exon 1b possesses TATA and CAAT boxes, but that giving rise to exon 1a does not [22], although it does contain GC-rich regions. Although the proximal region flanking the start of exon 1b is a strong functional promoter, the equivalent region flanking exon 1a is not. The murine lumican gene promoter possesses an unconventional TATA box, with the sequence TATCA located 27 bp upstream of the transcription start site [23], that might have weak affinity for TATA-binding proteins. It is, however, preceded by a GC-rich region that could facilitate recognition of the weak TATA box by using Sp1 binding. It therefore seems that the absence of a strong TATA box is a common feature of this LRR-protein subfamily, and that proximal Sp1-binding sites might commonly have a role in facilitating transcription.

Much less is known about the identity of repressor elements within the LRR-protein gene promoters, although such elements have been described in both the human biglycan [24] and decorin [25,26] promoters. Nothing is known about the transcription factors that bind to the repressor element of the biglycan gene promoter, whereas the function of the repressor element of the decorin gene has been associated with AP1 binding. In this subfamily of proteins, however, the PRELP gene repressor element is the first to clearly involve the interaction of Ets proteins. It is also of note that this repressor element can function equally well upstream of other promoters, as illustrated in the present study with the SV40 promoter. The SV40 promoter

also utilizes Sp1 binding in the initiation of transcription, so it is interesting to speculate whether an interaction between an Ets protein and Sp1 could be responsible for repression.

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