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Functional analysis of the human annexin A5 gene promoter: a downstream DNA element and an upstream long terminal repeat regulate transcription

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Human annexin A5 is a ubiquitous protein implicated in diverse signal transduction processes associated with cell growth and differentiation, and its gene regulation is an important component of this function. Promoter transcriptional activity was determined for a wide 5′ portion of the human annexin A5 gene, from bp −1275 to +79 relative to the most 5′ of several discrete transcription start points. Transfection experiments carried out in HeLa cells identified the segment from bp −202 to +79 as the minimal promoter conferring optimal transcriptional activity. Two canonical Sp1 sites in the immediate 5′ flanking region of a CpG island were required for significant transcription. Strong repressive activity in the distal promoter region between bp −717 to −1153 was attributed to the presence of an endogenous retroviral long terminal repeat, homologous with long terminal repeat 47B. The downstream sequence from bp position +31 to +79 in untranslated exon 1 was also essential for transcription, as its deletion from any of the plasmid constructs abolished activity in transfection assays. Electrophoretic mobility-shift assays, Southwestern-blot analysis and affinity chromatography were used to identify a protein doublet of relative molecular mass 35 kDa that bound an octanucleotide palindromic sequence in exon 1. The DNA *cis*-element resembled an E-box, but did not bind higher molecular mass transcription factors, such as upstream stimulatory factor or activator protein 4. The discovery of a downstream element crucial for annexin A5 gene transcription, and its interaction with a potentially novel transcription factor or complex, may provide a clue to understanding the initiation of transcription by TATA-less, multiple start site promoters.

Key words: DNA-binding proteins, gene regulation, promoter activity, repetitive elements, transcription factors.

INTRODUCTION

Annexin A5 harbours the essential tetrad structure and calcium-dependent phospholipid-binding properties that have made it a key model for studying the basic function(s) of this 12-member chordate gene family. Its anti-inflammatory, anticoagulant and growth-inhibitory actions stem from diverse properties that include calcium ion channel activity [1], inhibition of signal transduction enzymes, such as protein kinase C and phospholipase A_2 [2,3], avid calcium-dependent binding to anionic membrane phospholipids [4] or cytosolic protein targets [5], and interaction with extracellular matrix proteins [6–8]. Annexin A5 is abundantly and ubiquitously expressed [9], and is further inducible by phorbol esters [10], the c-*fos* oncogene [11], oestradiol [12] and gonadotropin-releasing hormone [13]. Its sensitivity to tissue developmental state and cellular growth conditions is manifested by accumulation in differentiated non-proliferating cells [14,15] and suppression in cancers [16], suggesting that its possible role in signal transduction is related to growth inhibition [13]. The observation that annexin A2 is frequently associated with the opposite changes emphasizes the need to understand the molecular mechanisms governing the differential regulation of individual annexin genes according to tissue specificity, growth state and responsiveness to external stimuli.

The annexin A5 gene occupies approx. 29 kb of human chromosome 4q27, and is comprised of an untranslated exon 1 plus 12 coding exons spliced congruently to annexin A11 and its respective descendents [10,17,18]. Structural comparisons of annexin A5 orthologues from human [10], rat [19], mouse [20] and chick [21] have helped to identify conserved regulatory motifs and coding regions with implied roles; however, the functionally active domains remain to be characterized. The localization of essential *cis*-acting genomic elements and the identification of *trans*-acting protein factors involved in annexin A5 gene expression are requisite to understanding the molecular control mechanisms for this and related genes, and may shed light on their possible involvement in tissue-specific growth and differentiation. Regulatory regions have been partially characterized in only three other annexin genes, i.e. annexin A1 in pigeon [22] and human [23–26] and those for human annexins A6 [26,27] and A7 [28]. Annexins A5 and A6 shared their most recent common ancestor 500–700 million years ago [20], yet they retain no apparent homology in noncoding regions. Although both gene promoters lack a TATA box or initiator element and have GC-rich regions containing putative Sp1 binding elements, annexin A5 exhibits marked differences in its initiation of transcription at multiple start sites and from an alternative, noncoding exon 1a in rat nervous tissues [19]. It thus falls into a special class of genes for which transcriptional control mechanisms remain poorly understood, hence the characterization of its promoter activity could have broad relevance.

MATERIALS AND METHODS

Plasmid constructions

A wide portion of the human annexin A5 gene promoter, from position −1275 to position +79 relative to the first transcription

Abbreviations used: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; tsp, transcription start point; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EST, expressed sequence tag; LTR, long terminal repeat; AP, activator protein; USF, upstream stimulatory factor; MED-1, multiple start site element downstream; NCBI, National Center for Biotechnology Information.

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start point (tsp1), was subcloned into plasmid vector pUC19. This subclone was used to generate deletions of the insert using a double-stranded, nested deletion kit (Pharmacia). Progressive 5′ deletions yielded gradually smaller promoter segments, that were excised from the pUC19 vector by double digestion with restriction enzymes *Hin*dIII and *Kpn*I. Individual promoter fragments were then ligated to reporter plasmid pGEM4Z-chloramphenicol acetyltransferase (CAT), provided by M. F. Young (see [29]), and derived from vector pGEM4Z (Promega) by ligation of the coding sequence of bacterial CAT. The human annexin A5 promoter-CAT plasmids obtained by this procedure were named pA5HP and pA5HP-1 to pA5HP-9. An additional 3′ deletion construct pA5HP-10 was obtained by the same procedure and spanned positions −1275 to −104. Other reporter plasmid constructs, pA5HP-D31 (bp +31 to +79) and pA5HP-D38 (−30 to +79), were obtained by partial restriction digestions with the enzyme *Hae*III, which cut quite frequently near the tsp. The remaining plasmids, pA5HP-NF, pA5HP-NFD31 and pA5HP-7D31, were obtained by PCR using the appropriate 17-mer primer based on their end sequences. All constructs were resequenced by the dideoxy chain-termination method [30] to ensure their fidelity with the published promoter sequence ([10]; GenBank® accession number U01681]. The reporter β-galactosidase plasmid P319US3-lacZ was a gift from A. M. Colberg-Poley (see [31]).

RNA preparation and primer extension analysis

Total RNA from HeLa cells transiently transfected with the plasmid reporter construct pA5HP-7 was isolated by the isothiocyanate/acid-phenol method [32] and 50 μg was used as template. The primer used was oligonucleotide 5′-TTTAGC-TTCCTTAGCTCCTGAAAAT-3′ specific to the CAT sequence; 100 pmol of ^{32}P-5′-end-labelled oligonucleotide and 50 μg of total RNA were preheated together at 90 °C for 2 min and annealed at 53 °C for 7 h. Extension proceeded at 37 °C for 2 h in the presence of 400 units of Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.), and the reaction products were separated by SDS/PAGE in a 6% denaturing gel which included a sequence ladder for size estimation of relative molecular mass.

Cell cultures and transfections

HeLa cells (A. T. C. C., Rockville, MD, U.S.A.) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1 mM L-glutamine, 11 units/ml penicillin and 0.1 mg/ml streptomycin sulphate. For transfections, cells were plated out at a density of 3×10^5 cells/well in six-well culture plates and grown for 12–16 h to 80–90% confluency. Solution A consisted of 0.5 μg of the reporter plasmid DNA in 100 μl of DMEM without serum and antibiotics, and solution B contained 4 μl of LIPOFECTAMINE™ (Gibco BRL) in 100 μl of DMEM without serum or antibiotics. These two solutions were mixed vigorously and incubated at room temperature for 30 min to allow DNA–liposome complexes to form, then 0.8 ml of DMEM was added to the mixture. Cells to be transfected were previously washed with medium lacking serum and were subsequently put in contact with the transfection mixture for 5 h. After this time, 1 ml of DMEM supplemented with 20% serum was added, and incubation proceeded for 24 h before restoring the initial medium. Cells were harvested for assay 48 h from the onset of transfection.

Protein extracts and enzyme assays

Whole cell extracts were prepared from transfected cells by washing twice with PBS, incubating for 5 min in TEN buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl), and scraping the plate. Cells were recovered by centrifugation at low speed, resuspended in buffer [15 mM Tris/HCl, pH 8.0, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.4 mM PMSF] and lysed by three cycles of freeze–thawing and vortex-mixing. After centrifugation of extracts to remove cell debris, the protein concentration of the supernatant was measured by the Bio-Rad protein assay (Bio-Rad Laboratories). The CAT enzyme assay was performed with a commercial kit, 'CAT-ELISA' (Boehringer Mannheim), according to kit instructions. β-Galactosidase activity was measured spectrophotometrically at 420 nm using *o*-nitrophenyl β-D-galactopyranoside as substrate. Relative CAT activity was corrected for transfection efficiency using β-galactosidase and normalized to a value of 100 arbitrary CAT units for the activity generated by construct pA5HP-NF included in all assays.

Electrophoretic mobility-shift assay (EMSA)

Nuclear protein extracts were prepared from HeLa cell culture monolayers at 90% confluency. Cells were washed three times with isotonic-buffered saline, scraped from 35 mm plates, collected by centrifugation and resuspended in 200 μl of buffer A (Hepes/KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT and 5 mM PMSF) containing a mixture of protease inhibitors (Boehringer Mannheim). Cell lysis proceeded during incubation in ice for 10 min. Nuclei were collected by centrifugation (14500 ***g*** for 20 s at 4 °C) and resuspended in buffer B (20 mM Hepes/KOH, pH 7.9, 0.2 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.8 mM DTT, 25% glycerol, 5 mM PMSF and protease inhibitor mixture). After incubation in ice for 20 min, the nuclear protein extract was collected in the supernatant following centrifugation at 14500 ***g*** for 5 min at 4 °C. Protein concentration was determined by the Bio-Rad protein assay. DNA–protein binding reactions were performed in a total volume of 50 μl, and consisted of a mixture of 10 μg of protein, 1 μg of poly(dI-dC) and 30000–50000 c.p.m. of [^{32}P]dCTP-labelled DNA probe in buffer C (9 mM Tris/HCl, pH 7.9, 9 mM $MgCl_2$, 0.6 mM EDTA, 0.6 mM DTT and 12% glycerol). For supershift experiments, 2 μl of upstream stimulatory factor (USF)1-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the reaction mixture. This was incubated for 30 min in ice, followed by 15 min at room temperature, then subjected to non-denaturing 5% PAGE. Gels were dried under vacuum and exposed to autoradiographic film.

Southwestern-blot analysis

The procedure followed published techniques [33]. Briefly, 50 μg of nuclear proteins were mixed with loading buffer, boiled for 3 min and resolved by SDS/PAGE in a 12% gel at 15 V/cm for 2 h. Proteins were electrotransferred from the gel to nitrocellulose membrane, which was immersed and rocked for 10 min at 4 °C in binding buffer (25 mM Hepes/KOH, pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM DTT and 6 M guanidinium chloride). The nitrocellulose was then transferred to binding buffer containing 3 M guanidinium chloride and incubated as in the previous step. Renaturation was achieved by eight successive washes in binding buffer, each with a 50% stepwise reduction of guanidinium chloride, and a final 1 h incubation in the absence of guanidinium chloride plus 5% non-fat milk powder and 5 μg/ml sonicated salmon sperm DNA. The membrane was then

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transferred to buffer containing 0.25 % non-fat milk powder and incubated at room temperature for an additional 30 min before adding the ^{32}P-labelled DNA probe and continuing the incubation for 2 to 12 h. After four 7 min washes at room temperature with binding buffer, the membrane was air-dried and autoradiographed.

Western-blot analysis

A duplicate lane containing nuclear extract proteins that were electrophoresed and transferred to the same nitrocellulose membrane used for Southwestern blots was utilized for Western-blot analysis. The membrane was blocked in 5 % non-fat dry milk/TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.05 % Tween 20) at room temperature for 1 h, incubated with anti-USF1 as primary antibody, diluted 1:3000 in TBST for 2–3 h and washed three times for 10 min each with TBS. The secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce Chemical, Rockford, IL, U.S.A.) was added to the membrane at a dilution of 1:5000 in TBS/5 % non-fat dry milk, incubated at room temperature for 1 h and then washed three times for 10 min each with TBS. Antigen–antibody complexes were detected using ECL® Western-blotting detection reagents (Amersham Pharmacia Biotech).

Affinity purification of sequence-specific DNA-binding proteins

The method used was a modification of published techniques [34]. The annexin A5 promoter DNA fragment from +31 to +79 was excised from plasmid pA5HP-D31 with restriction enzymes *Hin*dIII plus *Bam*H1, multimerized by ligation and end-labelled with Klenow enzyme in the presence of biotin-14dCTP (Life Technologies). Labelled DNA probe was then incubated with HeLa cell nuclear protein extracts under the same conditions as for EMSA, for 30 min in ice followed by 15 min at room temperature. Affinity purification of sequence-specific DNA-binding nuclear proteins used streptavidin MagneSphere paramagnetic particles (Promega) to bind DNA–protein complexes via biotin–streptavidin interaction. A magnetic separation stand permitted the isolation of proteins specifically bound to biotin-labelled DNA probe from other proteins in the extract. After washing four times with 1 ml of binding buffer (4.5 mM Tris/HCl, pH 7.9, 4.5 mM $MgCl_2$, 0.3 mM EDTA, 0.3 mM DTT and 6 % glycerol), the specifically bound proteins were eluted by a step gradient of 100 mM to 1 M KCl in binding buffer. Electrophoretic analysis of the eluted fractions was used to resolve the proteins bound to DNA and these were visualized by silver staining with the 'silver stain plus' kit (Bio-Rad).

Bioinformatic analyses

BLAST and QUERY e-mail servers were utilized for sequence database searches and retrieval respectively, from the National Center for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov. Programs from DNASIS (Hitachi Software Engineering, San Francisco, CA, U.S.A.) calculated the sequence G+C content, and Z-Hunt 2.0 [35] was used for Z-DNA detection. SIGNAL SCAN 4.0 [36] identified sequence matches to known DNA *cis*-regulatory elements in conjunction with the Transcription Factor Database (NCBI) and TRANSFAC database [37]. REPBASE 3.0 [38] was searched for genomic repetitive elements using the RepeatMasker program (http://ftp.genome.washington.edu/RM/RepeatMasker.html) developed by A. F. A. Smit and P. Green.

RESULTS

Initiation of transcription in the human annexin A5 gene

Primer extension analysis of annexin A5, using tissue RNA samples as template for reverse transcription, previously demonstrated a heterogenous pattern of extension bands [10,20], indicating that gene transcription may initiate at multiple sites. Plasmid constructs containing 5′ and 3′ serial deletions of the predicted promoter regulatory region were made to further assess the start site variability and to localize regions responsible for transcriptional activity. Promoter fragments fused to the coding sequence of CAT were transiently transfected into HeLa cells and the resulting transcriptional activity of individual plasmid constructs was measured. One construct, pA5HP-7, containing the region from positions −202 to +79 relative to the most 5′ tsp, was the minimal effective promoter sequence for optimal transcription. It generated CAT reporter levels of the same magnitude as other constructs with more extensive 5′ sequence, and produced transcripts with lengths clustered in several bands (Figure 1). A sequence ladder was used to calibrate these transcripts and define the most 5′ tsp at bp 1276 'Acgggccaa…' of the published genomic sequence [10]. Bands corresponding to other major transcriptional start sites were observed at bp positions +32 (tsp2), +55 (tsp3) and at bp +70 further downstream in exon 1 (Figure 1). These results were

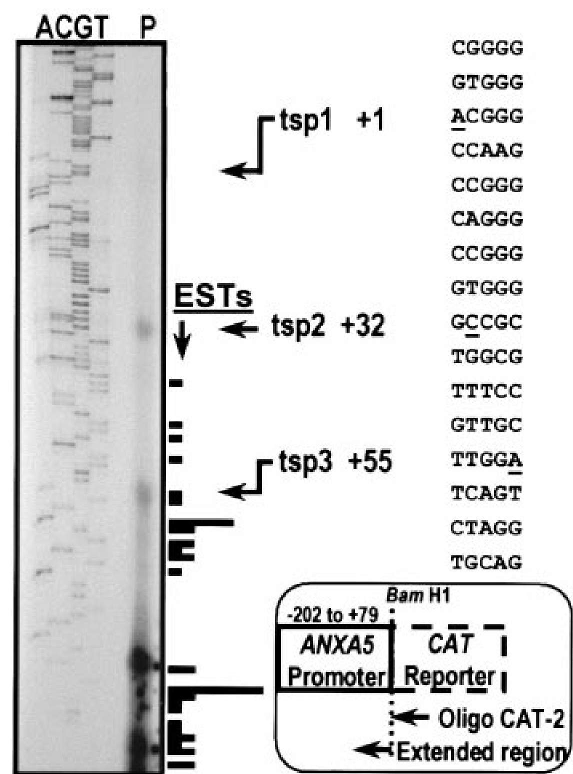

Figure 1 Primer extension analysis of the human annexin A5 gene (ANXA5)

A DNA sequence ladder (lanes ACGT) is aligned with gel bands corresponding to length(s) of primer extended products (lane P) obtained from total RNA of HeLa transfected cells with plasmid reporter construct pA5HP-7 and oligo CAT-2 as primer. The sequence origins of confirmed 5′ ESTs are aligned in a bar histogram to the immediate right of the gel. Exonic sequence on the right contains underlined nucleotides corresponding to the three indicated, major tsps. A structural outline of the transfected expression construct used to produce the RNA template is included (bottom right panel).

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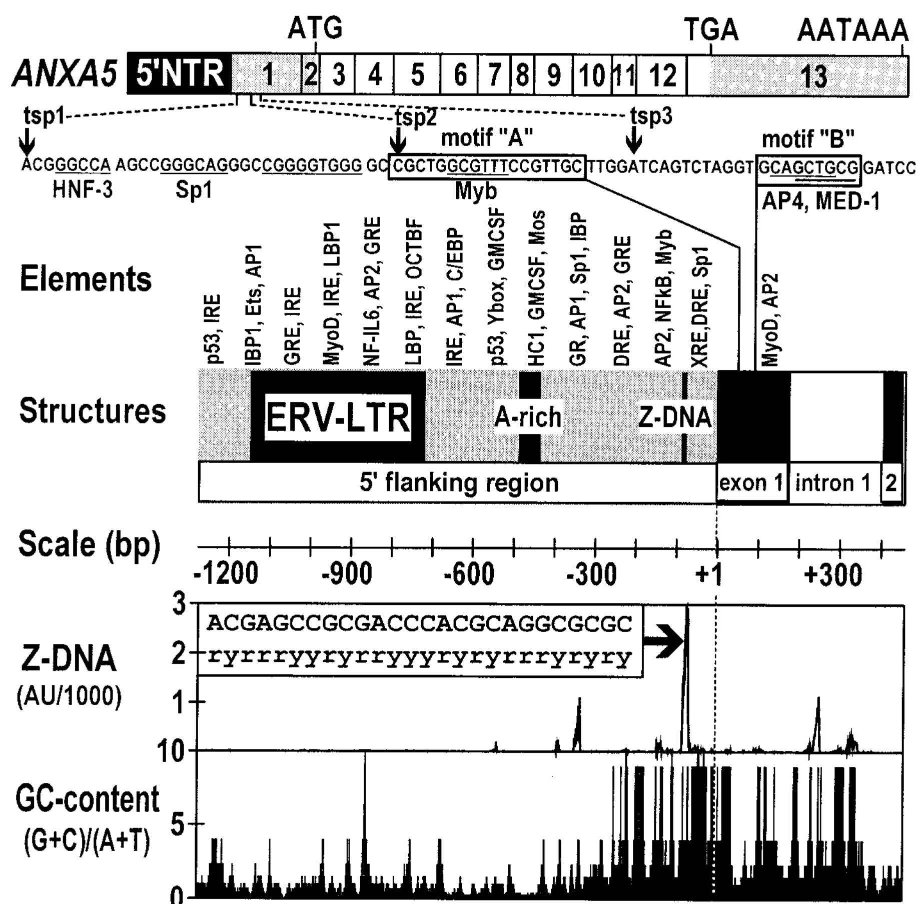

Figure 2 Structural features of the 5′ regulatory region in human annexin A5

The human annexin A5 gene structure (top panel) displays the upstream non-transcribed region (NTR) and the distribution of 13 exons consisting of untranslated (grey-shaded) and coding (open blocks) segments. The 5′ sequence of exon 1 is shown immediately below with putative transcription factor binding sites, study motifs and the initial tsps. Major structural elements comprising the 5′ flanking region include various potential regulatory elements identified by SIGNAL SCAN [36], a 437 bp endogenous retroviral (ERV) LTR homologous to LTR47B in REPBASE 5.0 [38], a 60 bp adenine-rich segment and a 25 bp stretch of Z-DNA, drawn to scale. Below the scale is a plot of promoter Z-DNA-forming potential in arbitrary units [35], with a sharp peak around −70 bp corresponding to the inset sequence. A plot of the (G+C)/(A+T) ratio over a 10 bp floating window (bottom panel) identifies a CpG island in the core promoter region, ±300 bp surrounding tsp1.

corroborated by ample evidence for annexin A5 transcription to expressed sequence tags in databases of expressed sequence tag (EST) (NCBI), with 5′ sequences commencing as early as +37 bp, appearing more frequently at +57 bp and inferred to originate near tsp2 and tsp3 in exon 1 (Figure 1).

Structural features of the human annexin A5 gene promoter

The design of deletion constructs for the human annexin A5 gene promoter used the tsp1 as a reference point and was guided by the location of potential *cis*-acting DNA regulatory elements and gross structural features in the 5′ flanking region (Figure 2). An extensive segment in the distal region was identified by RepeatMasker as a human endogenous retroviral long terminal repeat (LTR). It spanned 437 bp, from −717 to −1153 (Figure 2), and shared 69% nucleotide identity with the LTR47B consensus in REPBASE 3.0 [38] and up to 85% identity with homologous LTRs in current sequence databases. A 60 bp, adenosine-rich, low-complexity segment was located −454 to −513 bp upstream from the tsp. A CpG island with an average 75% G+C nucleotide content spanned the region 300 bp in either direction from tsp1 and contained the presumptive core promoter. A prominent, 25 bp Z-DNA segment of alternating purine/pyrimidine nucleotides was detected by the Z-Hunt-II program [35] 75 bp upstream of the tsp in both the human and chick annexin A5 genes.

Localization of transcriptional activity in the human annexin A5 gene

Transcriptional activity was determined for each of 16 CAT reporter constructs obtained by progressive terminal deletions from the human annexin A5 promoter region (−1275 to +79 bp) (Figure 3). In contrast to the full activity observed with region −202 to +79 bp in construct A5HP-7, a complete absence of activity in construct pA5HP-10 (−1275 to −104) identified the core promoter fragment between positions −103 to +79 as indispensable for transcription. Other constructs delineated several functionally important segments in the distal promoter region (−1077 to −739), the proximal upstream flanking region

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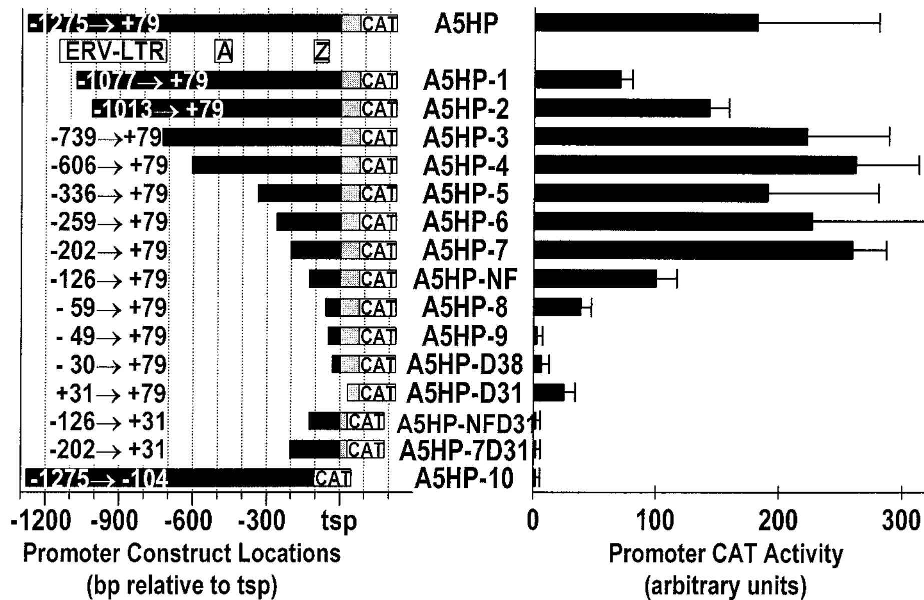

Figure 3 Construction and transcriptional activity of human annexin A5 plasmids transiently transfected into HeLa cells

DNA sequence fragments of the 5′ regulatory region (left panel) were included in the design of nested deletion constructs spanning −1275 bp of untranscribed 5′ flank (solid bars) up to +79 bp of transcribed exon 1 (grey bars). The right panel depicts means ± S.D. of transcriptional activity from three or more independent transfections with each of the corresponding plasmid constructs. CAT levels are expressed in arbitrary units for the mean relative to the normalized value of 100 for construct pA5HP-NF in each transfection experiment and corrected for transfection efficiency against β-galactosidase activity.

(−202 to −49) and the proximal transcribed region of exon 1 (+31 to +79). Progressive deletion of the first-mentioned segment from pA5HP-1 to pA5HP-2 to pA5HP-3, or its extension in the full-length construct A5HP, resulted in a gradual rise to maximal transcriptional activity (Figure 3). Deletion of the second active segment from construct pA5HP-7 to pA5HP-NF, pA5HP-8 and pA5HP-9 resulted in a significant decrease in transcriptional activity (Figure 3). The apparent positive regulatory influence of this region could be ascribed to several potential regulatory *cis*-elements [e.g. activator protein (AP) 2, nuclear factor κB, Myb, xenobiotic response element and nuclear factor W1). However, this isolated region (−202 to −49) lacked transcriptional activity, as other constructs associated with either additional 5′ or 3′ sequence (i.e. A5HP-10 and A5HP-7D31 respectively) had no activity. Finally, construct A5HP-8 (−59 to +79) established that the proximal transcribed region of exon 1 alone had appreciable transcriptional activity (Figure 3). Further 5′ deletions of 10 bp or 29 bp from A5HP-8 excised the two putative Sp1 sites in the immediate 5′ flanking region and resulted in a complete loss of activity in constructs pA5HP-9 or pA5HP-D38. The fact that constructs pA5HP-NFD31 and pA5HP-7D31 were devoid of activity, whereas A5HP-NF and A5HP-7 containing a proximal extension of exon 1 from bp +31 to +79 had significant activity, implied that positive regulatory elements in this downstream region were essential for full promoter activity.

Nuclear protein binding to pA5HP-D31 fragment

The 49 bp segment of exon 1 in construct pA5HP-D31 (+31 to +79) was indispensable for gene transcription, and its activity was presumably mediated by an interaction of a *cis*-binding element(s) in the construct with one or more cellular nuclear factor(s). We sought to identify a *trans*-acting protein factor able to bind this 5′ gene segment and retard its migration in EMSA. The corresponding DNA insert was ^{32}P-end-labelled and incubated with nuclear extracts of either HeLa or HL60 cells. The migration of this fragment was significantly retarded in the presence of nuclear extract from either cell type, apparently due to the formation of major DNA–protein complexes (Figures 4A and 4B). The specificity of the interaction was determined by adding 50-, 100- or 200-fold excess of non-radiolabelled DNA fragment, either identical or unrelated, and observing that only the specific probe displaced radiolabel from the top band using either HeLa or HL60 cells. The protein composition of the complexes was investigated by Southwestern analysis of nuclear proteins obtained from HeLa cells. Nuclear protein extracts were electrophoresed, transferred to nitrocellulose membrane, renatured and incubated with the ^{32}P-labelled DNA fragment from the construct. This DNA specifically bound one or two protein bands with electrophoretic mobility near the 35 kDa marker (Figure 4C), whereas a radioactive probe of unrelated DNA did not form any detectable bands.

DNA affinity purification of *trans*-acting protein factors

The identification of 35 kDa protein(s) bound to the D31 DNA fragment required their purification prior to characterization or microsequencing. This was accomplished by using streptavidin MagneSpheres to bind the biotin-labelled pA5HP-D31 insert. Nuclear protein(s) that specifically and avidly bound the DNA were thus retained by the MagneSphere matrix and, after thorough washing of the MagneSpheres with buffer alone, were eluted by a stepwise gradient of KCl in buffered solution. A typical elution profile (Figure 5A) confirmed the presence of two protein bands very similar in mass, but partially separated by a discontinuous salt gradient. Their similar size and salt-dependence during elution made it difficult to distinguish whether

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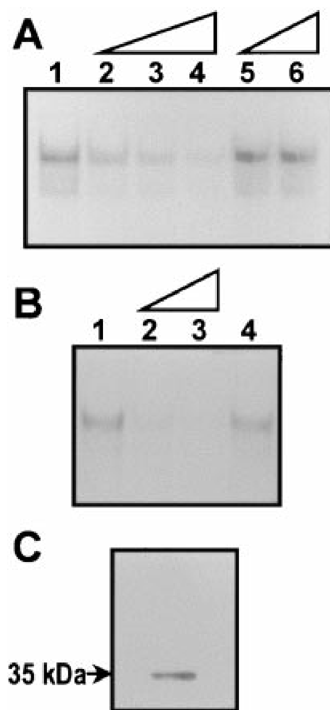

Figure 4 EMSA and Southwestern detection of specific DNA–protein binding

A radiolabelled fragment D31 (bp +31 to +79) in construct pA5HP-D31 was incubated with nuclear extracts from HeLa or HL60 cells, followed by electrophoresis in 5% polyacrylamide under non-denaturing conditions. (**A**) DNA–protein complexes in the upper band formed from HeLa cell extract competed with 50-, 100- and 200-fold excess of identical, unlabelled DNA (lanes 2–4), or with a 100- or 200-fold excess of unrelated DNA (lanes 5 and 6). (**B**) Similar results were obtained with HL60 cells without competitor (lane 1), with a 100- or 200-fold excess of identical, unlabelled DNA (lanes 2 and 3) or with a 200-fold excess of unrelated DNA (lane 4). (**C**) Autoradiograph of a Southwestern analysis of HeLa cell nuclear protein extract using DNA fragment D31 as probe to show the bound 35 kDa nuclear protein(s).

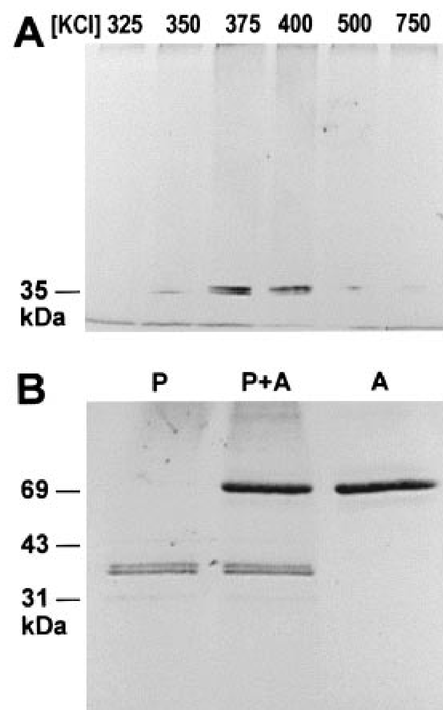

Figure 5 Affinity purification of nuclear protein(s) bound to DNA fragment D31

(**A**) D31 fragment (bp +31 to +79) was labelled with biotin-14dCTP and incubated with HeLa cell nuclear proteins. The mixture was exposed to streptavidin-bound MagneSphere beads to trap the biotin-labelled DNA together with bound protein. The magnetically isolated bead–DNA–protein complex was washed to remove non-specifically bound proteins, after which specifically bound protein was eluted by a stepwise gradient of KCl, electrophoresed in a 12% polyacrylamide gel and visualized by silver staining. Protein aliquots eluted at 325 mM, 350 mM, 375 mM, 400 mM, 500 mM and 750 mM KCl (lanes 1–6 respectively). (**B**) An aliquot of the protein sample eluted by 375 mM KCl was incubated with bacterial alkaline phosphatase and the mixture electrophoresed in a 20% polyacrylamide gel (lane P + A) beside the untreated protein (lane P) and alkaline phosphatase alone (lane A). Enzyme treatment did not change the mobility of the 35 kDa protein doublet.

these were distinct proteins or isoforms of the same protein post-translationally modified, for example, by phosphorylation. Treatment of the salt-eluted proteins with bacterial alkaline phosphatase produced no electrophoretic change that would have suggested a difference in phosphorylation state (Figure 5B). Electrophoresed proteins were transferred to PVDF membranes (Millipore) for microsequencing, but apparent N-terminal blockage thwarted attempts to obtain partial peptide sequence for comparison with known transcription factors.

Sequence delimitation of the *cis*-elements interacting with nuclear proteins

The DNA sequence within the D31 DNA fragment was further scrutinized using SIGNAL SCAN to identify prospective elements that might be responsible for specific binding of the isolated 35 kDa proteins. Two main regions of interest in exon 1 were designated DNA motifs A and B (Figure 2). Motif A exhibits 70% nucleotide conservation among orthologous annexin A5 genes and harbors a potential binding site for c-MYB (GCGTTT). Motif B contains a palindromic sequence of 8 nt (GCAGCTGC) with possible binding elements for AP4 (CAGCtg) or USF (CANNtg as E-box), and extending to another resembling multiple start site element downstream (MED-1; GCTCCG), for which the binding protein has not been identified. Both motifs were investigated individually by EMSA and Southwestern analysis. EMSAs were performed with HeLa nuclear protein extracts plus radiolabelled motif A or motif B, both of which caused significant band retardation (Figures 6A and 6B). Only the band corresponding to the protein interacting with motif B disappeared in the presence of excess cold DNA, whereas protein interaction with DNA motif A appeared non-specific, since the retarded band was not displaced with up to a 200-fold excess of the same unlabelled DNA fragment. A difference in the results shown in Figures 4 and 6 suggests that the presence of motif B together with A in the D31 fragment can prevent the nonspecific binding of A observed in Figure 6. These results implicated sequence motif B in a direct and specific interaction with protein(s) in the retarded band complex. Therefore the chromatographically purified nuclear proteins previously observed to associate with the D31 fragment (Figure 5) were analysed by Southwestern blots, with the individual DNA motifs as probes. The results in Figure 6(C) clearly confirmed that only DNA motif B interacted with the protein(s) eluting at 375 mM KCl, whereas no interaction was observed with either DNA motif A or a DNA fragment consisting of a polylinker fragment of the pBSK plasmid vector.

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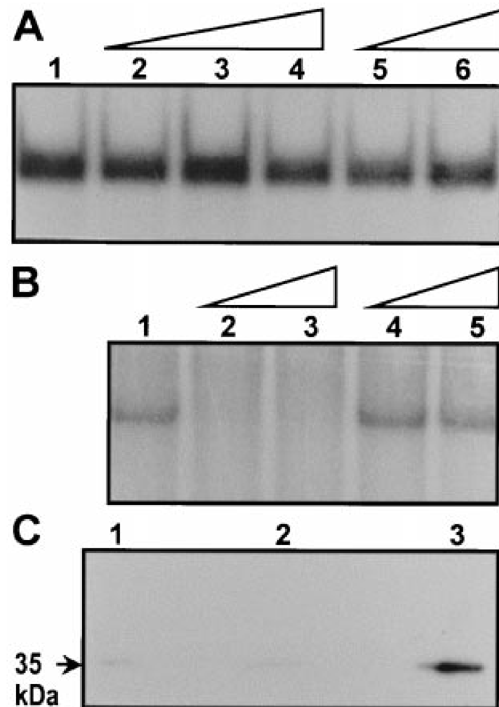

Figure 6 Delimitation of the DNA element which binds to 35 kDa nuclear protein(s)

EMSA was performed with HeLa nuclear protein extracts and radiolabelled DNA identical with either motif A or motif B. Both DNA elements were retarded by nuclear proteins during electrophoresis. (**A**) The protein interaction with motif A was unaffected by a 50-, 100- or 200-fold excess of unlabelled motif A (lanes 2–4) or 100- to 200-fold excess of unrelated DNA (lanes 5–6). (**B**) Radiolabelled motif B was displaced specifically by an excess of unlabelled motif B DNA (lanes 2 and 3) but unaffected by unrelated DNA (lanes 4 and 5). (**C**) Southwestern-blot analysis showed negligible binding between the affinity-purified 35 kDa nuclear protein sample eluted at 375 mM KCl and either pBluescript polylinker (lane 1) or motif A (lane 2), in contrast with the strong interaction with radiolabelled DNA motif B (lane 3).

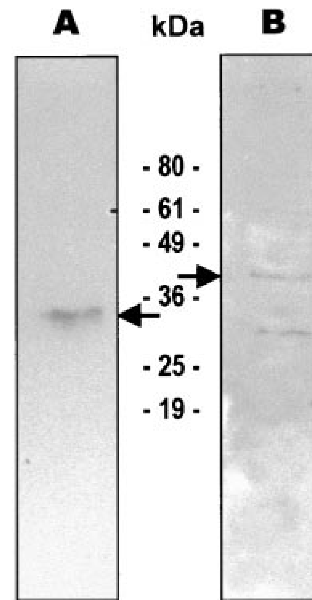

Figure 7 Distinction between motif B-binding proteins and USF1 by size and immunoreactivity

Duplicate lanes containing equal amounts of nuclear protein extract were electrophoresed, transferred to the same nitrocellulose membrane and cut separately. (**A**) One lane was subjected to Southwestern analysis using ^{32}P-labelled D31 DNA fragment to detect 35 kDa binding protein (lower arrow). (**B**) Western analysis of the duplicate lane utilized a USF1-specific antibody to detect a 43 kDa protein (upper arrow).

Attempt to identify motif B-binding nuclear protein(s)

Motif B may contain an E-box (CANNTG) known to bind USF1 and USF2 [39], which are associated with cell cycle control [40] and growth inhibition [41]. Although an updated consensus (CACGtg) compiled from 12 genes in TRANSFAC 4.2 was inconsistent with motif B, we sought evidence for USF interaction with motif B. Bandshift experiments, such as those described in Figures 4(A) and 4(B), utilized a USF1-specific antibody. No supershift was observed in the presence or absence of antibody (results not shown), indicating the absence of protein–DNA complexes. Western-blot analysis (Figure 7B) verified that anti-USF1 antibody recognized the expected 43 kDa protein and others of lower molecular mass in the nuclear extracts, but none coinciding with the 35 kDa protein(s) with affinity for the D31 DNA fragment. Southwestern analysis (run in parallel) of nuclear protein extracts incubated with the [^{32}P]D31 DNA fragment confirmed the specific interaction with 35 kDa protein(s) (Figure 7A) having mobility distinct from the 43 kDa USF. The motif B sequence was also compatible with AP4 binding, but the 48 kDa molecular mass of this protein, and similar negative results from EMSA, using the specific radiolabelled DNA fragment together with recombinant AP4, also excluded AP4 as the candidate factor (results not shown).

DISCUSSION

We obtained a regional profile of human annexin A5 promoter activity to identify the core transcriptional unit and to implicate both upstream genomic elements and downstream-factor-binding sites in its modulated expression. The abundance and ubiquity of annexin A5 expression has been well documented and a survey of EST databases substantiated this. More than 800 annexin A5 cDNA transcripts among 4.6 million ESTs from hundreds of human and mouse tissue libraries ranked it fourth in incidence among annexins, after A2, A11 and A1 [9], approx. 10 times more than the calculated gene 'average', with predominant expression in placenta and ovary. Numerous 5′ ESTs supported the tsp designations revealed by primer extension. Although mRNA and protein stability may contribute to these levels of expression [14], the importance of transcriptional control for annexin A5 was first recognized when it was found to be a major inducible target of c-*fos* proto-oncogene [11], presumably mediated via AP1-compatible binding sites present in the 5′ flanking region and intron 1 [10]. Although c-*fos* is commonly associated with enhanced cell proliferation, its induction may also be associated with the cessation of cell division [11], depending on both the balance of other regulatory factors, the composition of AP1 heterodimer, and its own multiple gene targets. The fact that it induces both annexins A2 and A5, which exhibit differential actions with respect to protein kinase C, calcium-dependent phospholipid affinity and cell growth, makes it conceivable that annexins participate in some of these contrasting effects of c-*fos* in different cellular contexts. The elevation of annexin A5 during quiescent cell states, its repression during cell proliferation and its growth inhibitory properties [13,14], emphasize the need to identify genomic repetitive elements, *cis*-

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DNA elements and *trans*-acting protein factors responsible for this regulated expression.

Progressive 5′ deletion of the core promoter (−202 to +79) effectively diminished transcriptional activity (Figure 3), and the ultimate exclusion of putative Sp1-binding sites immediately upstream of the tsp in A5HP-9 (−49 to +79) totally abolished activity. The vital, positive role of this proximal 5′ flanking region in annexin A5 transcription makes it a key target for regulatory control by external stimuli through the interaction of protein transcription factors with responsive elements, including AP2, nuclear factor κB, c-*myb*, xenobiotic response element and nuclear factor W1 (Figure 2). In the absence of a TATA box, basal transcription is likely to be controlled by the Sp1 sites in the immediate 5′ flanking region, although full activity clearly requires additional upstream determinants. These Sp1 sites lie in the middle of a CpG island spanning bp −300 to +300 bp and close to a potential Z-DNA-forming segment (Figure 2), which signify both compositional and conformational changes influencing the genomic landscape, the accessibility of *cis*-acting elements and the activation of gene transcription [35].

Divergence in structure and specialization in gene regulation are the principal means by which gene families selectively adapt and functionally diversify during evolution of the organism. The variable abundance and tissue-specific expression of the 12 paralogous human annexins are distinguishing features that have evolved since the proliferation of these genes in primitive chordates 500–800 million years ago [42,43]. This has been achieved by evolutionary selection for advantageous mutations and by the introduction of interspersed, repetitive genomic elements, such as endogenous retroviruses and Alu 'master' elements that can disrupt, usurp or endow regulatory control of host gene expression [44]. The annexin A5 distal promoter hosts a solitary LTR that decreases the basal activity of promoter constructs containing it, presumably due to chromatin remodelling and/or by its ample contribution of candidate *cis*-elements [e.g. nuclear factor induced by interleukin 6 (NF-IL6), γ-interferon response element, glucocorticoid response element, AP1, AP2, myogenic determination factor, octamer-binding factor, leader-binding protein-1 and interferon (element) binding protein-1] that could bind available transcription factors. It is one of thousands of conserved LTRs estimated to exist in animal genomes, especially abundant in simians and mice [45] and frequently located in the vicinity of transcribed genes [46]. Based on the given LTR mutation rate of 0.2–0.3%, its greater than 15% difference from other known LTRs suggests that it may be more than 50–75 million-years-old and therefore present in primates and possibly other mammalian annexin A5 genes. The contribution of LTRs as structural mutagens and donors of functional DNA elements for transcription factor binding, such as c-*myb* [47] or steroids [48], could directly impinge on annexin A5 regulation (Figure 2), although the *in vivo* relevance of specific domains must be assessed under defined conditions. The prospect that human annexin A5 transcription is subject to retroviral repressor element(s) (Figure 3) is a concrete example of symbiotic co-evolution at the molecular level, and a potentially major modulator during cell cycling, tissue differentiation or changed cellular environment. Interestingly, a LINE-2 element with transcriptionally repressive components has been characterized in the upstream promoter of annexin A6 [27], with which annexin A5 shared its most recent common ancestor [43]. A complete murine endogenous retroviral element (MuERV)-L resides in mouse annexin A5 intron 4 [20], and a rare Alu subfamily retroposon at bp −452 to −627 in the human annexin A1 promoter [24], further testify to the involvement of retroposons in the structural evolution of annexin genes.

The possibility that elements downstream of the most 5′ tsp (e.g. hepatocyte nuclear factor-3, Sp1, c-*myb*, AP4, myogenic determination factor and AP2) were also involved in annexin A5 transcription arose from analogous studies of other genes [49]. The important experimental observation that exon 1 segment +31 to +79 was absolutely essential for transcriptional activity of any 5′ flanking construct (A5HP-NFD31, A5HP-7D31 and A5HP-10; see Figure 3) was especially intriguing. A focused study of this region identified conserved motif A with a c-*myb* element and motif B containing an AP4 site with similarity to MED-1 and a variant E-box element possibly binding USF. However, the specific binding of 35 kDa HeLa nuclear proteins to the D31 fragment and to isolated motif B clearly differentiated this interaction from one involving either MYB (70 kDa), AP4 protein (48 kDa on SDS/PAGE) or USF (43 kDa), by size and binding criteria. Some similarity of the motif B sequence with MED-1 was potentially relevant because no transcription factor has yet been described for this element, which purportedly defines a distinct class of RNA polymerase II, TATA-less promoters featuring multiple initiation sites [50]. However, the 35 kDa relative molecular mass of the protein doublet, the uniqueness of the motif B binding element itself, and recent uncertainty about the role of MED-1 elements in transcriptional control [51], suggest that this might represent a novel interaction for transcriptional regulation. The presence of similar, downstream elements in other annexin genes raises the possibility that this interaction may have more widespread relevance in gene transcriptional regulation. Initial attempts to characterize further the implicated transcription factor(s) have been hampered by N-terminal blockage that precluded direct peptide sequencing; however, internal peptide microsequencing of additional purified products, molecular cloning, or testing of novel 35 kDa candidate transcription factors, should eventually reveal the nature of this functionally significant binding interaction.

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