Divergent Structure of the Human Synexin (Annexin VII) Gene and Assignment to Chromosome 10

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ABSTRACT: The human synexin (annexin VII) gene occurs as a single copy at chromosome 10q21.1-21.2 and substantially deviates in size and in the location of splice junctions from the other two well-characterized members of the annexin gene family, lipocortin I (annexin I) and calpactin I (annexin II). The synexin gene contains 14 exons, including an alternatively spliced cassette exon, and spans approximately 34 kb of DNA. Only five of the fourteen splice junctions are conserved compared to other annexins, and the differences are particularly pronounced in the exons that encode the C-terminal third and fourth conserved repeats in the gene product. Although parallels between exons and protein domains were not apparent, we did observe clustering of splice junctions corresponding to either the unique N-terminal domain or the conserved C-terminal tetrad repeat domain, which is common to all annexins. Furthermore, a complete analysis of the 5′ flanking region of the annexin VII gene revealed an entirely different set of cis-acting and enhancer elements compared to other annexin genes. We conclude that the annexin VII gene may have arisen by a divergence from the evolutionary pathway taken by both annexins I and II.

Synexin (annexin VII) is an intracellular, calcium-dependent, phospholipid-binding protein (Burns et al., 1989; Magendzo et al., 1991; Pollard et al., 1990; Pollard & Rojas, 1988; Creutz et al., 1978, 1979), which drives not only chromaffin granule aggregation (Creutz et al., 1979) but also the fusion of granule membranes (Creutz, 1981; Stutzin, 1986; Nir et al., 1987) and liposomes (Hong et al., 1981). The mechanism of membrane fusion may involve the formation of a "hydrophobic bridge" of synexin molecules between the fusing membrane partners (Pollard et al., 1991). Synexin also forms voltage-dependent calcium channels in natural and artificial membranes (Burns et al., 1989; Pollard et al., 1990; Pollard & Rojas, 1988) and has been implicated in the process of calcium-dependent exocytosis (Pollard et al., 1990, 1991; Creutz, 1992). Synexin shares a homologous C-terminal tetrad repeat with the other members of the annexin gene family, and the unique N-terminal domain is distinguished from that of other family members in its large and highly hydrophobic character (Burns et al., 1989; Pollard et al., 1990; Pollard & Rojas, 1988). The conserved C-terminal domain may be the locus of the membrane-spanning channel structure in synexin and other annexins, since other annexins such as endonexin II (annexin V; Rojas et al., 1990) and lipocortin I (annexin I; Pollard et al., 1992) also exhibit channel activity.

Exon/intron boundaries have been mapped for human, mouse, rat, and pigeon lipocortin I (Hitti & Horseman, 1991; *vide infra*), mouse calpactin I (Amiquet et al., 1986), and human annexin III (Tait et al., 1993). In these cases, the splice junctions have been shown to be identical in most locations within both the unique N-terminal domains and the conserved C-terminal tetrad repeats. Thus, the high degree of conserved sequence observed in all members of the annexin gene family is mirrored at the level of organization of the gene for at least three members of the family. We therefore anticipated that the conserved region of the synexin gene might also be similar in organization to the two annexins already known.

However, we report here that the gene for synexin is in fact substantially different in structure from the lipocortin I and calpactin I genes previously described. The 34-kb synexin gene is much larger, due in part to the additional exons needed to encode the lengthier N-terminal domain. Furthermore, while the locations of the intron/exon boundaries in the synexin gene for the hinge region and for repeat 1 and most of repeat 2 are identical, or at least very close, to equivalent boundaries in the genes for annexins I and II, most other splice junctions deviate profoundly in other parts of the synexin gene, corresponding to repeats 2–4. We discuss these results in the context of the origin and possibly divergent evolutionary pathways within the annexin gene family. We also found that the synexin gene occurs in a single copy, localized to chromosome 10. Finally, we defined the introns flanking the cassette exon responsible for the unique form of synexin mRNA found in brain, heart, and skeletal muscle (Magendzo et al., 1991).

MATERIALS AND METHODS

Genomic Southern Analysis. High molecular weight human liver DNA was prepared as previously described (Maniatis et al., 1989). Ten micrograms of this DNA were then digested with several restriction endonucleases, and the products were separated on 1% agarose gels. The DNA was then blotted onto nitrocellulose membranes and baked to ensure fixation. DNAs from several species were processed in a similar manner after digestion with *Sac*I (Clontech, Inc.) and used as a "zoo blot". Sources of DNA included human placenta, rhesus monkey, Sprague-Dawley rat, BALBc mouse, dog, cow, rabbit, and yeast (*Saccharomyces cerevisae*). Membranes were hydridized with a randomly primed, 1.5-kb

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cDNA probe prepared by digestion of the *anx*VII[1] cDNA with *Pst*I and *Sst*I and then incubated overnight at 65 °C. Membranes were then washed in 0.1× SSC/0.1% SDS for 30 min at 65 °C, and autoradiography was carried out for 3–6 days.

Isolation of Genomic Clones. The same 1.5-kb restriction fragment from the human *anx*VII cDNA described above was used to screen a human genomic library prepared in the λ-FIX vector (Stratagene, Inc.) from lung fibroblast DNA, partially digested with *Sau*3AI. The phage library was plated on susceptible bacteria, and nitrocellulose filter lifts were prepared. Hybridization of the filters with the random-primed probe was carried out overnight at 42 °C. The filters were then washed at 50 °C for 60 min in 0.1× SSC/0.1% SDS. Twelve positive clones were identified and purified through three rounds of screening. Purified phage DNA was digested with *Sac*I endonuclease prior to Southern analysis with both *anx*VII cDNA and synthetic oligonucleotide probes from different regions of the *anx*VII cDNA.

Of the twelve original clones, four were identified as independent overlapping clones, and these were used to initiate a walk along the chromosome in the 5′ direction. For this purpose, a size-selected DNA library was prepared in the λ-dash vector system, as follows. Human liver genomic DNA was prepared and digested with *Sac*I restriction endonuclease. The 12-kb fragments were separated by gel electrophoresis, purified with glass beads using the Geneclean kit (BIO 101 Inc., La Jolla, CA), and ligated into the λ-dash vector. An oligonucleotide probe from the second exon (primer 1, dAAGCCACTAGGATAAGGATAC TGACCAGAAGG) was then used to screen 10^6 recombinant phages. Further walking in the 5′ direction was performed using a human peripheral blood leukocyte library in λ-gem (Promega) and a human placenta genomic DNA library in λ-FIX (Stratagene). The oligonucleotide probes were prepared from nonrepetitive intron sequences of the λ-11 clones.

Determination of Exon/Intron Boundaries and Analysis of Regulatory Elements. Restriction endonuclease fragments from the different genomic clones were subcloned into the pBS KS$^+$ vector (Strategene, Inc.) or into the pGX2627 vector in the appropriate restriction sites. The recombinant subclones were sequenced by the dideoxy method (Sanger et al., 1977) using cloned T7 Sequenase (United States Biochemical Corp.) and flanking vector primers or primers from the *anx*VII cDNA. Oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer Model 380B. Splice junctions were identified by detailed comparison of genomic and cDNA sequences.

Except for 21 bases in exon 1, the nucleotide sequences in the other exons exactly correspond to the nucleotide sequence previously reported for the R10/R16 *anx*VII cDNA clone (Burns et al., 1989). Nucleotides 1–21 of the R10/R16 clone were therefore replaced by sequences from clone F4 (Magendzo et al., 1991), since these were identical to the *anx*VII genomic DNA. Furthermore, 28 additional bases at the 5′ end of exon I have been inferred from analysis of the *anx*VII mRNA. Thus, the estimated size of exon I is 87 base pairs.

Putative regulatory elements contained in the 5′ upstream region of the *anx*VII gene were analyzed using a proprietary computer-based search method.

PCR of Genomic Sequences. Since none of the above cosmid libraries contained the 3′ end of the *anx*VII gene, we decided to identify splice junctions in this 6-kb region using PCR analysis of genomic DNA. PCR reactions were performed using 1-μg aliquots of human genomic DNA as templates and oligonucleotide probes corresponding to cDNA sequences flanking specific sides of each intron. The primers used in determining the 3′ end of the gene were the following:

primer A:
(p900, dATT TGG ACG AGA CCT TGA AAA GGA CAT)

primer B:
(rp 1090, dTGT TAA AGC AAG ATT CAT CGG TCC C)

primer C:
(p985, dCGT GAT GAG AAC CAG AGT ATA AAC C)

primer D:
(p1300, dCAT CTG TGC CAG CAC CTT TCA TAG)

primer E:
(p1251, dCTT CTT TGC TGA GAG GCT CTA CT)

primer F:
(rp2014, dCTT TCT TAA AAG GTG CCA CAG GTA)

primer G:
(rp1774, dAGA GAT TGC TGT GAT TTT TAT TCA)

Other primers were prepared from exon sequences flanking each intron.

Each PCR reaction mixture of 100-μL volume contained 25 pmol of each primer, 0.2 mM each dNTP, 2.5 units of *Taq*I polymerase (Perkin-Elmer Cetus), PCR buffer (50 mM KCl, 15 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3), and 0.1% (w/v) gelatin. After initial denaturation for 10 min at 94 °C, samples were subjected to 35 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 55 °C, and polymerization for 1 min at 68 °C. The PCR fragments were filled in by a final extension step of 7 min at 68 °C. Amplified DNA was then separated on 1% or 4% Nusieve agarose gel (FMC Bio Products) and subcloned into a TA cloning vector (Invitrogen Corp.). The identity of each DNA band was verified by hybridization to an internal oligonucleotide sequence and/or by sequencing. To determine the presence and size of introns, the size of the amplified DNA was compared to the known distance between each primer site on the cDNA.

Preparation of RNA. Total RNA and poly(A) RNA were prepared from human liver as described (Maniatis et al., 1989), using the guanidine thiocyanate method and oligo(dT) chromatography. Poly(A) RNA was prepared from the cultured human skin fibroblast cell line CCD-19SK (American Type Culture Collection), by using the Fast Track mRNA isolation kit (Invitrogen, Inc.).

PCR Amplification from Poly(A) RNA. Poly(A) RNA, in aliquots of 1 μg in 20 μL, was used as a template in cDNA synthesis reactions. Each reaction included 10 pmol of the antisense primer (rp140: dGCG GCC GCG TCG ACG GAA AAG ATG ACT CCT GAC CTG CA). The incubation mixture included the PCR buffer described above, 10 units of RNasin (Promega Corp.), 200 units of MLV reverse transcriptase (Bethesda Research Labs), and 1 mM each dNTP. The reverse transcriptase reactions were incubated for 1 h at 37 °C. The subsequent PCR reaction, in the same buffer, included 10 pmol of sense primer and 2.5 units of *Taq*I polymerase. The sense primers from the 5′ end used in these reactions were the following:

[1] Abbreviations: Anx, annexin gene product; *anx*, annexin gene or mRNA; SDS, sodium dodecyl sulfate.

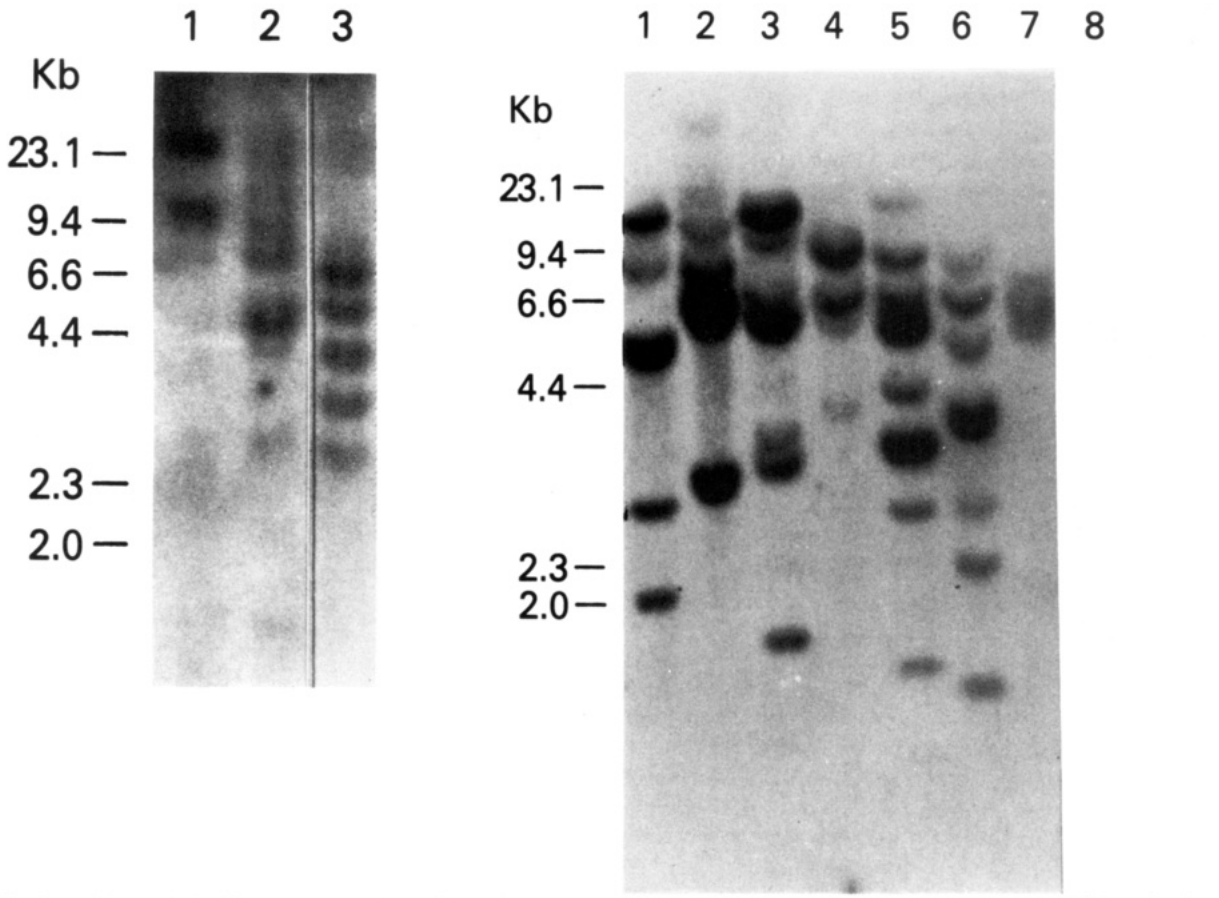


FIGURE 1: Southern blot analysis of human DNA. (A, left) Human DNA digested with different restriction endonucleases. High molecular weight human liver genomic DNA was prepared as described in Materials and Methods (37) and cut by the following restriction endonucleases: lane 1, *Eco*RI; lane 2, *Hind*III; and lane 3, *Pst*I. DNA samples (10 μg) were run on a 1% agarose gel and hybridized with a full-length synexin cDNA probe from clone R10/R16. After a stringent wash of 30 min in 0.1× SSC/0.1% SDS at 65 °C, the blots were subjected to autoradiography. (B, right) Phylogenetic survey of the synexin gene. Genomic DNA from the following species were digested with *Sal*I: lane 1, human; lane 2, monkey; lane 3, rat; lane 4, mouse; lane 5, dog; lane 6, cow; lane 7, rabbit; lane 8, yeast. Blots were hybridized and washed as described for A.

primer 6:
(dATC TTG CGG GAG ACC GGG TTG GGC T)

primer 7:
(dGAA CGC CCC GCC CAC CCT GGG CCC G)

primer 8: (dCGG GCT CGC GAG ACG)

primer 9: (dTCC GGG GGT GTT CCC GGC TCG AG)

Subsequently, 30 cycles of denaturation were performed for 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 2 min at 72 °C, and a final extension extension step of 7 min at 72 °C. The reaction products were separated on 3% Nuseive/1% Seakem agarose gels.

Primer Extension Analysis. Primer extension analysis was performed as described previously (Srivastava et al., 1990). Briefly, two synthetic antisense oligonucleotides were prepared on the basis of sequence data from the second exon and were used for the extension of human liver RNA. These were primer 1 (rp172) and primer 10 (rp114, dAGG ATA TCC AGG GAA A). The extension reaction products were separated on a sequencing gel. In parallel, we ran an M13 sequencing reaction to provide size markers.

Somatic Cell Hybrids and Filter Hybridization. Human and rodent parental cells, fusion procedures, and isolation and characterization of hybrids were as previously described (McBride et al., 1982). In general, hybrids were tested for the presence of each human chromosome (except the Y) by isozyme, karyotypic, and Southern hybridization analyses. Seven of the human–hamster hybrids were positive, using 29 primary clones and 13 subclones. The human–mouse hybrids consisted of 18 primary hybrids and 35 subclones, of which 4 were positive. High molecular weight DNA isolation, agarose gel electrophoresis, and Southern hybridization were performed by standard procedures (McBride et al., 1989).

In Situ Hybridization. Metaphase spreads were prepared using peripheral blood lymphocytes from a normal male (46, XY) after BrdUrd synchronization, as previously described (Gnarra et al., 1990). Chromosomal DNA on slides was denatured in 0.07 N NaOH/70% ethanol (Singh et al., 1977) and hybridized with the tritiated *anx*VII cDNA 3′ end (Harper & Saunders, 1981). Following autoradiography, the spreads were treated with 0.25% Wright stain diluted in phosphate buffer (pH 6.8) and photographed. The slides were destained, stained with Hoecht 33258 (150 g/mL) in deionized water for 30 min, rinsed, air-dried, mounted in 2× SSC, and illuminated for 30 min with a UV lamp. Metaphases were rephotographed after restaining in Wright stain (Bhupendra, 1988).

RESULTS

Southern Blot Analysis of Restriction Fragments of Genomic DNA. Our initial step in the analysis of the *anx*VII gene was to estimate its size from the sizes of restriction fragments of human genomic DNA. We purchased high molecular weight human liver DNA digested with restriction endonucleases (*Eco*R1, *Hind*III, and *Pst*I) and detected the differently sized products with a 1.5-kb fragment of the *anx*VII

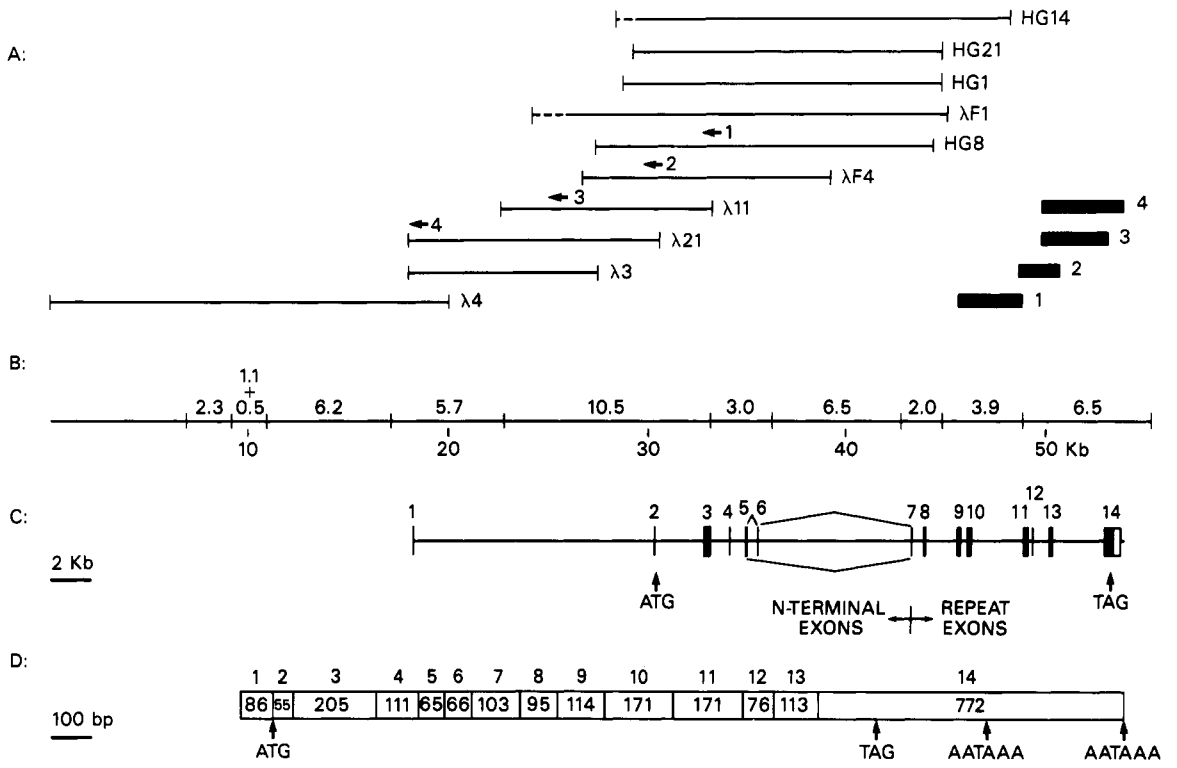


FIGURE 2: Structure of the human synexin gene and the nature of clones and probes used in its determination. (A) Size and distribution of clones and probes. Descriptions of abbreviations are given in the text. Solid bars are PCR fragments of the 3′ end of the gene. Arrows are the locations of synthetic oligonucleotide primers used as probes for walking along the chromosome in the 5′ direction. Thin lines represent inserts of overlapping λ clones. (B) *Sac*I restriction map of the human synexin gene. Numbers above the line indicate fragment sizes in kb. Numbers below the line indicate a genomic scale in kb. The complete gene is *ca.* 34 kb. The first fragment from the left does not have a natural *Sac*I site at its 5′ end. The third fragment (1.1 and 0.5) is really two fragments whose order was not determined. (C) Primary mRNA transcript. Exons are indicated by solid boxes or vertical lines and are numbered. The alternatively spliced exon (2) is number 6, and the two pathways of expression are as indicated. The open boxes in exons 1 (not visible at this magnification) and 14 represent the 5′ and 3′ untranslated regions, respectively. Exons for the unique N-terminal domain and the conserved C-terminal tetrad repeat are separated in exon 7 and are delineated by the double-headed arrow. (D) Exon structure of mature synexin mRNA. The scale is larger than that for the previous sections. Numbers above the exons indicate the exon number. Boxed exons have a length proportional to their length, shown by numbers within the box. Arrows at the 3′ end indicate the two alternative polyadenylation signals in the mRNA. The locations of the ATG start codon and TAG stop codon are as indicated.

cDNA (**1**). As shown in Figure 1A, strongly hybridizing bands were apparent, with sizes ranging from 1.5 to 23 kb. The sums of the these sizes were in the range of 25–30 kb, indicating that the synexin genomic sequences could occupy a minimum length of approximately 30 kb.

Genomic DNA from human and various other species, including monkey, rat, mouse, dog, cow, rabbit, and yeast, were digested with the endonuclease *Sac*I and hydridized with the same *anx*VII cDNA probe as above. As shown in Figure 1B, specific labeled bands were detected on all of the mammalian DNA samples, and a product of *ca.* 10 kb was detected in the yeast DNA sample. Thus, the apparently large size of the human synexin gene appears to be sustained in other species, although the present data are less compelling in the cases of mouse, rabbit, and yeast.

Isolation of anxVII Genomic Clones. Efforts to isolate genomic clones for human *anx*VII were initiated by screening a λ-FIX library from human fibroblasts. As described in Materials and Methods, four clones were identified as independent, overlapping clones by Southern analysis with *Sac*I restriction endonuclease. These four clones (HG14, HG21, HG1, and HG8 in Figure 2) encompass 20 kb of the synexin gene and were consistent with the concept that synexin might be encoded by a single-copy gene. As will be shown here, this proved to be a correct interpretation.

To test whether these four clones represented the full gene, we hydridized each clone with oligonucleotide probes corresponding to different parts of the cDNA (see primers A–G in Materials and Methods). This analysis revealed that additional exons remained to be isolated. Specifically, the four clones above contained part of the first intron extended in a 3′ direction to exon 10. Splice junctions in *Sac*I fragments from the λ clones were sequenced with specific *anx*VII primers following subcloning in pBS KS$^+$ and pGX2627 (see Materials and Methods for details). This analysis revealed that the second exon started with the initiation codon, which was preceded by intron 1, of at least 3 kb in length.

Further Isolation of Synexin Genomic Clones by 5′ Walking. To isolate the 5′ end of the human synexin gene, we prepared oligonucleotide probes progressively more 5′ to each subsequently identified clone. We used probe 1 (see panel A of Figure 2 for numbered arrow) to identify F4, and F1 from a human lung fibroblast library in λ-FIX (Figure 2A). Since these clones extended the sequence in intron1 only another 2 kb, we continued the chromosome walking by cloning a specific 12-kb *Sac*I fragment, previously shown to hybridize to exon 2 probes. One positive clone, 11, allowed us to extend our walk an additional 5 kb farther into the first intron at the 5′ end of the gene, without yet reaching the first exon.

Our next approach was to sequence portions of the first intron in the 11 clone to identify nonrepetitive sequences. From these data, we were able to synthesize probe 3 (see Figure 2A) for screening of a human peripheral blood library. Two new clones, 21 and 3, were isolated (see Figure 2A) and shown to contain the 5′ noncoding region of the cDNA near a *Sau*3AI

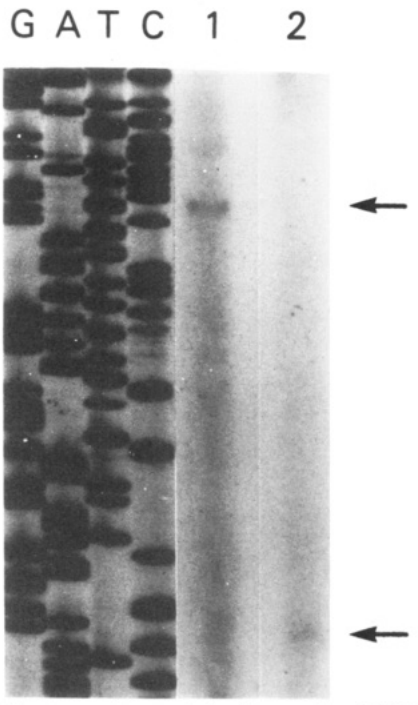


FIGURE 3: Primer extension analysis of human liver RNA. Antisense oligonucleotide primers to sequences in exon 2 (primer 1) or exon 3 (primer 10) were labeled with ^{32}P and hybridized to 50 μg of total RNA from human liver. The control RNA sample was tRNA. Extension was carried out using AMV reverse transcriptase, as described in Materials and Methods, and the reaction products were electrophoresed on a sequencing gel. The primer-extended product was obtained with primer 1 and is shown in lane 1. A similar product obtained with primer 10 is shown in lane 2. A nonrelated sequencing reaction served as a size marker ladder.

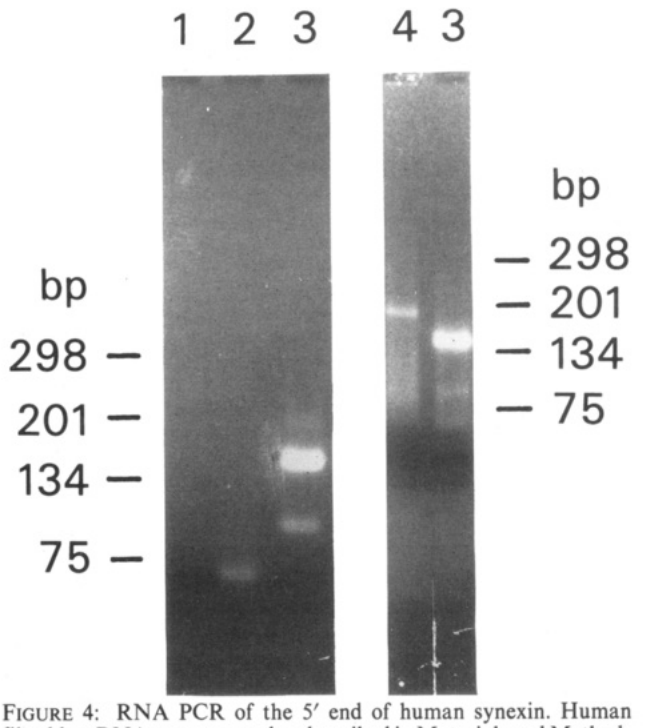


FIGURE 4: RNA PCR of the 5′ end of human synexin. Human fibroblast RNA was prepared as described in Materials and Methods in the presence of methylmercury, and 1 μg of RNA was used as the template for cDNA synthesis using MMLV reverse transcriptase. The PCR reaction was performed using the following sets of primers: lane 1, primers 5 and 9; lane 2, primers 5 and 8; lane 3, primers 5 and 6; lane 4, primers 5 and 7. The sequences of the primers are shown in Figure 5, where they are marked by a labeled arrow under the appropriate line. The products of the reaction were separated by electrophoresis on a 3% Nuseive/1% Seakem agarose gel. Examples from two separate experiments are shown. Commercial weight markers were a 123-bp ladder (BRL Life Technology, NY).

site just before the start of transcription (see Figure 2).

To isolate more upstream regulatory sequences, we used probe 4 from the 5′ end of clone 21 to screen a human placenta library in λ-FIX (see Figure 2A). We isolated 4, a 20-kb clone, which contained another 18-kb upsteam of clone 3 (see Figure 2A).

Isolation of 3′ Genomic Clones of anxVII by PCR. Since none of the above-mentioned *anx*VII genomic clones corresponded to exons 11–14, defining repeats 3 and 4 of the protein, we used the PCR method with several oligonucleotides prepared from the 3′ end of the cDNA. Four overlapping PCR fragments (see filled bars in Figure 2A) were cloned into pTA plasmids, and the remaining splice junctions were determined by sequencing. These data, taken together with the previous clones, yielded the entire human synexin gene of 34 kb, enclosed within 55 kb of genomic sequence.

Structural Features of the 5′ End of the anxVII Gene by Primer Extension Analysis and RNA PCR. In order to characterize the 5′ end of the *anx*VII gene and to locate transcriptional elements, we cloned a 3.0-kb *Sac*I fragment of 4 into the plasmid pBS (Strategene, Inc.). This fragment contained exon 1, flanked by 1.2 kb of upstream sequences and 1.8 kb of intron 1. For primer extension analysis, we used two antisense oligonucleotide primers, 1 and 10, which start 113 and 55 bp, respectively, downstream from the ATG initiation codon. Each primer was used to promote a separate primer extension reaction, resulting in one distinct band. Primer 1 gave rise to a 201-base fragment, indicating a start site at a G residue at a position 87 bases upstream from the ATG. Primer 10 gave rise to a 153-base fragment, indicating a start site at a C residue at a position 98 bases upstream of the ATG (Figure 3). These results therefore suggest the presence of two potential transcription initiation sites.

As a primer extension study could be affected by premature termination or by anomalous distances from the TATA or CCAAT boxes, we chose to test this conclusion further by RNA PCR analysis using human fibroblast RNA as the template. Primer 5 is located 46 bp downstream from the ATG, while primers 6–9 are located in different regions of the 5′ noncoding genomic sequences of the synexin gene. As shown in Figure 4, when primers 5 and 6 were used, a 140-bp product was obtained (Figure 4, lane 3). This is the expected size if the two oligonucleotides are aligned tandemly along the mRNA. Primers 5 and 7 yield a 170-bp product (Figure 4, lane 4), while primers 5 and either 8 (Figure 4, lane 1) or 9 (Figure 4, lane 2) did not yield a PCR product. These results indicate that primers 8 and 9 do not represent sequences belonging to the messenger RNA molecule. In addition, RNAse protection assays with *in vitro* transcribed RNA hybridized to poly(A) RNA yielded similar results, with a start site eight bases farther in the 5′ direction. These results are consistent with the transcription initiator site being located 87 bp upstream of the ATG (Figure 5).

Intron/Exon Boundaries of the anxVII Gene. Exon-containing genomic fragments and their flanking intron sequences were subcloned into plasmid vectors and sequenced using cDNA or vector-specific oligonucleotides. One of the first results was an understanding of the disparity between reported nucleotides 1–21 of the cDNA R10/R16 (Burns et al., 1989) and the equivalent sequence in genomic clone F4 (Magendzo et al., 1991). This analysis indicated that the sequence of nucleotides 1–21 of the cDNA R10/R16 is inverted relative to the sequence of clone 3 (bases 35–61, Figure 5) and is most likely a cDNA cloning artifact. Thus, on the basis of the identity of the F4 cDNA clone previously

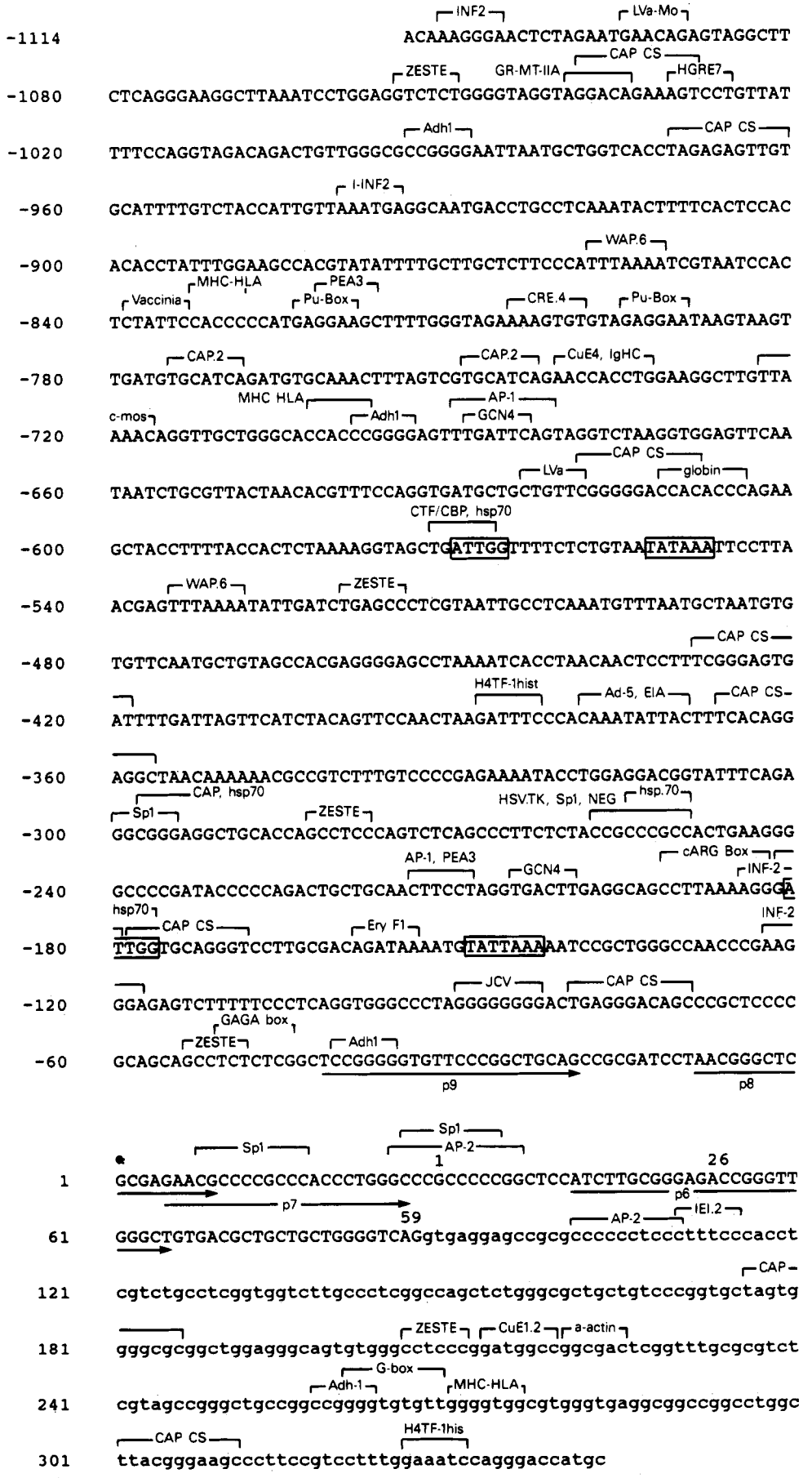


FIGURE 5: Nucleotide sequences of the 5′ end of the human synexin gene. Sequences of the 5′ flanking genomic DNA are numbered from −1 to 1114. The transcription initiation site is marked with an asterisk (*), and numbers up to 28 designate sequences of exon 1 in genomic DNA. Numbers 1–59 specify sequences corresponding to cDNA clones F4 and R10/R16, as explained in Materials and Methods. The sequence of the first intron is in lower case letters. Sequences similar to consensus sequences for transcription factors are marked by a line above the sequence. Sequences of the different oligonucleotides used in the RNA PCR analysis (see Figure 4) are indicated by an arrow under the appropriate line. Putative TATA boxes and CCAAT boxes are identified by a bar.

Table 1: Exon/Intron Structure of the Human Synexin Gene[a]

no.	5′ splice sites			3′ splice sites
1.	GCGAGA	------87------	59 GGTCAG^gtgagg	---12,000---- aaacaaaaaactacagagaaaaaactcttgttgttacag^
2.	AATGTC MetSe	-------55------	114 TATCCT^gtaagt TyrPro	----2,500----- ctatgatcgtatttgtttaaaaattattctgatttttag^
3.	CCACGT ProAla	------205------	319 CCGGAG^gtgagt roGlyV	-------900---- aaagacgttactactcgtacagcaatgttcttttctctag^
4.	TTCCTC alProP	------111------	430 TACCTG^gtaggt euProG	-------600---- aatttcatgattgaccttgttgccgtaattttagatatag^
5.	GTGGCT lyGlyP	-------65------	495 AGTCAG^gtgtgt SerGln	-------460---- ttcccatttttacctttataacttttcactcttttttcag^
6.	ATCAAT IleAsn	-------66------	AGTGAA^gtgagt SerGlu	----7,700---- ggaaatgctcgacaacggattttatgccttttatttttcag^
7.	CCTGCC ProAla	------103------	598 GTTTTG^gtgagg lyPheG	-------550---- ctaaacagattcaaaacactgtgtgtgtgtctctctcacag^
8.	GGACAG lyThrA	-------95------	693 GGCAAG^gtatgt GlyLys	----1,500---- gagaacgcgatatacaatgcttgtatttgtgattaaatag^
9.	GATTTA AspLeu	------114------	807 ATGCAG^gtacta MetGln	-------220---- ttaaacaaaaacaaacatattacaaccaagtaaagacag^
10.	GGAGCA GlyAla	------171------	978 TGCCAG^gtgagt CysGln	----2,800---- ctattatttatttcttccaacctgcctttctctttgcag^
11.	GGAAAT GlyAsn	------171------	1149 TCTAGG^gtatga SerArg	-------130--- tcggctttatctgatattcgttgctttaattttgtttgcag^
12.	ATGGCT MetAla	-------76------	1225 CCATCT^gtaaga hrIleL	-------870---- gagatcatgattccatgtctcaaccatctctaacttgcag^
13.	TGCAGT euGlnC	------113------	1338 AGTGAG^gtgagg SerGlu	----2,500---- cacagatgtttttctgacagaaatattatttcttatgcag^
14.	ATTGAC IleAsp	------772------	2110 AGACTT	

[a] The table should be read from left to right and then continue with the next line. The number of exons is given in the first column. The first and last six nucleotides of each exon are in upper case letters. Amino acids encoded by these bases are shown underneath in the three-letter amino acid code. In cases of codons split by intron sequences, the three-letter word is shared between two successive exons. The number of nucleic acids immediately 5′ to the splice site is indicated above the codons. Intron sequences are indicated in lower case letters. Putative branch sites conforming to the consensus YNYYRAY (Green, 1986) are underlined and were identified within a region 18–40 bp upstream of the 3′ intron/exon boundaries. Exon and intron sizes are given in base pairs.

isolated from the human fibroblast library (Magendzo et al., 1991), and genomic DNA from 3 in the 5′ noncoding region, we conclude that the first exon of the synexin gene is represented by 28 bp, inferred from the primer extension analysis, 21 bases from the F4 cDNA, and by nucleotides 22–59 of cDNA clone R10/R16 (Burns et al., 1989).

Intron/exon boundaries were determined (see Figure 1), and they conform to the consensus sequences established for intronic donor and acceptor splice signals (Breathnach & Chambon, 1981; Ohshima & Gatoh, 1987; Padgett et al., 1986). As shown in Table 1, each splice donor begins with GT, and each acceptor site ends with an AG and is preceded by a poly(pyrimidine)tract. We also detected the pre-mRNA splicing branch point consensus (YNYYRAY) in the region 18–40 bp upstream of the 3′ end of all synexin introns, except for intron 1 (see the underlined intron sequences in Table 1). In five of the introns (5, 6, 11, 13, and 14) there is one departure from consensus, while in two others (2 and 9) there are two departures. Introns interrupt the coding sequence in only two of the three reading frames. Interruptions between codons (class O) occur in eight cases (exons 2, 5, 6, 8, 9, 10, 11, and 13), while interruptions between the first and second bases of the codon (class I) occur in four cases (exons 3, 4, 7, and 12). However, no interruptions in the *anx*VII gene are observed between the second and third bases of the codon (class II).

Exon/Intron Sizes and General Features of the Synexin Gene. The sizes of each intron were estimated by comparing restriction and sequence analysis of subcloned fragments of the λ clones, or of PCR products of genomic DNA, with data from the *anx*VII cDNA. We also performed additional restriction mapping to determine the sizes of long introns (data not shown). As indicated above, the first exon is untranslated and is separated by a long 12-kb intron from the second exon (see Figure 1B,C and Table 2).

The second exon begins with an adenosine just prior to the translation initiation site ATG. Exons 2–6, which comprise most of the sequences coding for the unique N-terminal domain of AnxVII, are clustered within a 5.2-kb genomic segment and are separated from exons 7–14 by another large intron (intron 6) of 7.7 kb. The latter exons encode some of the hinge region of the N-terminus, as well as the conserved tetrad repeat domain of AnxVII. Thus, the separate unique and conserved domains in the gene product also seem to be sequestered from one another at the genomic level. Exon 14

Table 2: Segregation of Synexin with Human Chromosome 10[a]

chromosome no.	gene/chromosome +/+	+/−	−/+	−/−	% discordancy
1	4	7	24	60	33
2	4	7	19	65	27
3	6	5	30	54	37
4	10	1	49	35	53
5	5	6	20	64	27
6	7	4	33	51	39
7	6	5	32	52	39
8	10	1	18	66	20
9	6	5	27	57	34
10	11	0	0	84	0
11	8	3	22	62	26
12	8	3	22	62	26
13	5	6	23	61	31
14	7	4	36	48	42
15	4	7	42	42	52
16	6	3	28	56	33
17	8	3	50	34	56
18	0	11	37	47	51
19	4	7	25	59	34
20	7	4	34	50	40
21	7	4	56	28	63
22	4	7	23	61	32
X	8	3	41	43	46

[a] Discordancy represents the presence of the gene in the absence of the chromosome (+/−) or the absence of the gene despite the presence of the chromosome (−/+). The percentage discordancy is the sum of these numbers divided by the total number of hybrids examined, multiplied by 100. The human–hamster hybrids contained 29 primary clones and 13 subclones (7 positive of a total of 42). The human–mouse hybrids represented 18 primary clones and 35 subclones (4 positive of a total of 53).

contains 120 bp of coding sequence, a TAG stop codon, and an additional 648 bp of noncoding sequence. Two alternatively used polyadenylation signals at positions 1753 and 2049 result in the production of two mRNAs of 1.8 and 2.1 kb, respectively (2).

The first intron contains a Z-DNA motif 308 bp upstream of the 3′ end splice junction. This is represented by a stretch of 42 bp of almost perfectly alternating purines and pyrimidines, which is typical of a sequence able to form the Z-DNA structure: 5′-GT GT GT GT GT GT GT GT GT GT GT GT T GT GT AT GT GT GT GT AT AT-3′. This sequence is interrupted once and is underlined at that site. The sequence is deserving of note, if only due to the fact that since Z-DNA structures are found in a large number of transcriptional enhancers from a variety of RNA and DNA tumor viruses (Nordheim & Rich, 1983) and are correlated with the regulation of transcription in other genes, it seems plausible to postulate a similar role in the synexin gene. In addition, the above $(dG–dT)_n$ interspersed element is an example of short tandem repeats (i.e., microsatellites), which are frequently polymorphic and may be useful genetic markers (Weber & May, 1989).

The sequence upstream of nucleotide 1 has features that are typically found in promotor regions, and these are shown in Figure 5. This sequence is generally enriched in GC pairs, which have been shown to be associated with Sp1 binding sites. Indeed, several potential Sp1 binding sites are located within the 5′ end of the gene and are marked in Figure 5 at positions −255 and −300 bp upstream of the start site and in exon 1 at positions +8 and +25. The 5′ upstream region also contains two potential TATA box and reverse complement CCAAT box elements. One pair is placed 140 bp upstream of the putative transcription initiation site, and the other is farther upstream of this site at −553 bp. However, although the distances between the TATA box and CCAAT box in each set fall within the typical range of distances between these two motifs, their locations relative to the initiation start site are farther upstream than expected. This may be an idiosyncracy of the synexin gene or, alternatively, may indicate that there could be another untranslated exon, otherwise hidden from our view, which would place the start of transcription closer to the CAAT box.

Chromosomal Assignment of the Human Synexin Gene. DNAs isolated from human–mouse somatic cell lines and their parental cell lines were examined for the presence or absence of the human synexin gene by Southern blot techniques. A single band (19 kb) was detected in *Eco*RI digests of human DNA from the peripheral blood leukocytes of 10 unrelated individuals, and it was well resolved from cross-hybridizing rodent bands (see Figure 6A,B). Southern blots were prepared after the digestion of DNAs from 10 unrelated individuals with 12 different restriction endonucleases (*Eco*RI, *Hind*III, *Bam*HI, *Xba*I, *Sac*I, *Tac*I, *Msp*I, *Pvu*II, *Pst*I, *Bgl*II, *Eco*RV, and *Kpn*I). No restriction polymorphisms were detected by hybridization with the synexin 3′ cDNA probe. Analysis of the entire panel of hybrids permitted unambiguous assignment of this gene to human chromosome 10 (see Table 2), and the gene segregated discordantly (>20%) with all other human chromosomes.

Further localization of the *anx*VII gene was obtained by *in situ* hybridization of human metaphase spreads (Figure 7). A total of 104 metaphases containing 244 grains were analyzed, and 45 grains (18.4% of total) were found on chromosome 10. Nineteen (19) of the 45 grains (42%) on chromosome 10 were clustered on band q21 (see Figure 7C).

DISCUSSION

The data reported here show that the synexin gene occurs in a single copy of 14 exons and 13 introns and spans approximately 34 kb of DNA. We have also noted that splice junctions in the gene form two general clusters corresponding to either the unique N-terminal domain or the conserved C-terminal tetrad repeat domain common to all annexins. A similar division occurs in the genes of at least two other known members of the annexin gene family: human, pigeon, mouse, and rat lipocortin I (annexin I) and mouse calpactin I (annexin II). However, as we will detail further, the size and the specific organization of splice junctions for the human synexin gene deviate substantially from the two other known annexin genes, particularly for repeats 3 and 4.

Comparison of the Synexin Gene Structure with Other Annexin Genes in the N-Terminal Domain. The annexin gene family presently consists of 13 members, but only a few, in addition to synexin, have been studied intensively. As indicated above, all annexins are distinguished from each other in having a unique N-terminal domain. Synexin and annexin XI differ from all of the others by having a much larger and more hydrophobic N-terminal domain. Furthermore, exons 2–4 in synexin have no counterparts in annexins I or II. However, within the more proximal portion of the N-terminal domain, where analogous exons exist among annexins, synexin also differs from the other annexins by having an alternatively spliced casette exon (exon 6). Annexin VI also has a cassette exon, but it is located in the seventh repeat in the conserved C-terminal domain (Moss & Crumpton, 1990). Although the function of the cassette exon in synexin is not known, it is preferentially expressed in brain, heart, and skeletal muscle (Magendzo et al., 1991), where the larger molecule may have modified activity or subcellular localization. The cassette exon domain in synexin does occur in a location that has been identified in other annexins as a "hinge" region. For example, in AnxII, this domain is sensitive to proteolysis, which can

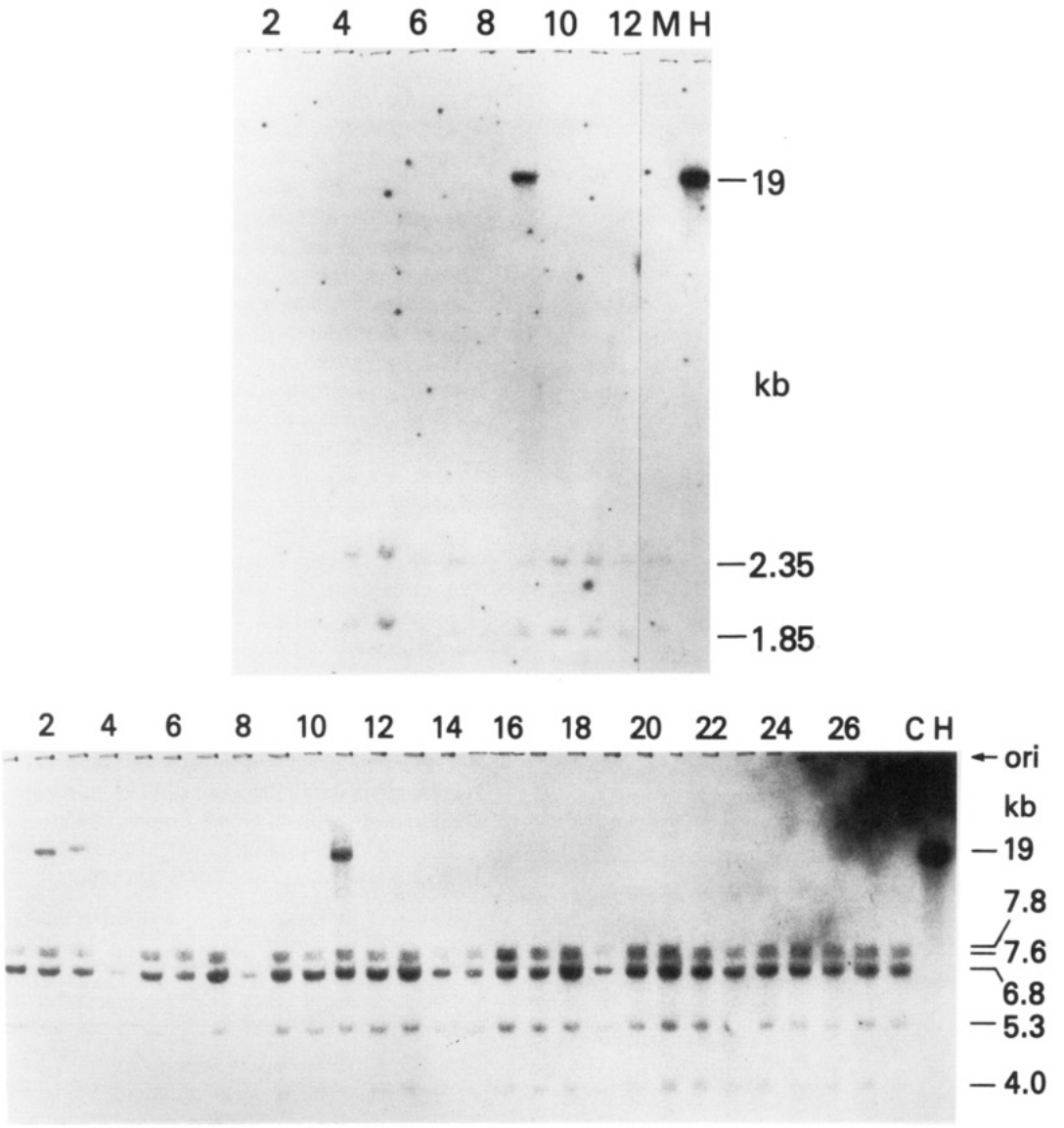


FIGURE 6: Chromosomal analysis using hybrid cells. Southern hybridization of representative *Eco*RI-digested human–mouse (A, top) and human–hamster (B, bottom) hybrid cell DNAs with a 0.9-kb human *anx*VII 3′ cDNA probe. Each lane contains a different hybrid cell DNA. Parental Chinese hamster (C), mouse (M), and human placental (H) DNAs are also shown along the margins. Lanes containing the human bands are approximately 19 kb in length; the sizes of cross-hybridizing rodent bands are indicated. The hybridizing human band and human chromosome 10 are only present in lane 9 (A) and in lanes 2, 3, and 11 (B).

result in an inhibition of actin binding or interaction with the small subunit p11 (Johnsson et al., 1988). This domain also has sites for phosphorylation by tyrosine kinase, protein kinase C, and pp60^{src} [summarized in Gerke (1992)]. In AnxI, this domain is phosphorylated by EGF receptor/kinase (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987) and by protein kinase C (Summers & Creutz, 1985; Khanna et al., 1986; Schlaepfer & Haigler, 1988). However, AnxVII does have potential phosphorylation sites recognized on the basis of sequence.

Within the proximal, shared N-terminal domain, the intron/exon junctions for exon 6 in synexin and exon 2 in both calpactin I and lipocortin I occur within nine residues of each other (see Table 3). Thus, in spite of the unique cassette exon in synexin, this would appear to be at least one example of a possible correlation between the location of splice junctions and a specific protein domain shared among at least three representatives of the annexin gene family.

Comparison of the Synexin Gene Structure with Other Annexin Genes Encoding the Conserved C-Terminal Domain. The conserved C-terminal tetrad repeat in synexin and other annexins is that part of the protein which binds calcium, interacts with phospholipids, forms calcium channels, and, where observed, fuses membranes (Pollard et al., 1990, 1991). Therefore, we anticipated that at the very least the locations of the splice junctions would be conserved in the parts of the gene encoding the conserved domains. This expectation is born out in the cases of lipocortin I and calpactin I, which share nearly identical splice junctions. However, the synexin gene has only five splice junctions in common with the other annexins. The details of the differences are as follows.

As shown in Table 3, the first repeat in synexin contains two exon/intron boundaries (7 and 8). These are reported to occur in identical positions and phases in both calpactin I and lipocortin I genes (boundaries 3 and 4, respectively). In the second repeat, boundaries 9 and 10 in synexin exactly correlate in position and phase with boundaries 5 and 7 reported for both the calpactin I and lipocortin I genes. However, within the second repeat, both calpactin I and lipocortin I genes reportedly share an exon/intron boundary 6 that has no parallel whatsoever in the synexin gene.

The deviation becomes more substantial in the third repeat, where synexin has two exon/intron boundaries, 11 and 12. Boundary 11 in synexin occurs six residues away from a splice

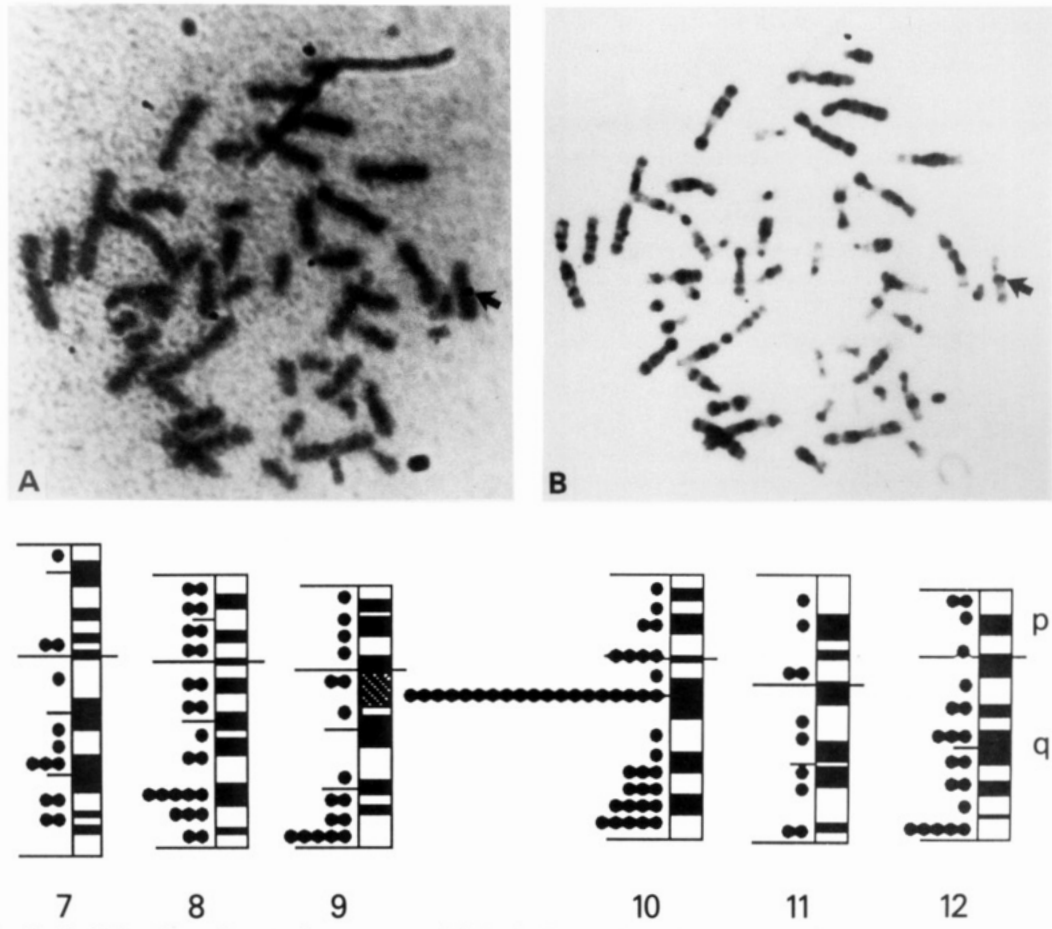


FIGURE 7: *In situ* hydridization to human chromosomes. (A) Typical metaphase showing a grain on chromosome 10 (arrow). (B) Same metaphase spread as in A, after replication banding showing the location of a grain on 10q21 (arrow). (C, bottom) The distribution of grains on chromosome 10 and some other "C" group chromosomes is shown. The grains were randomly distributed except for the cluster at 10q21.

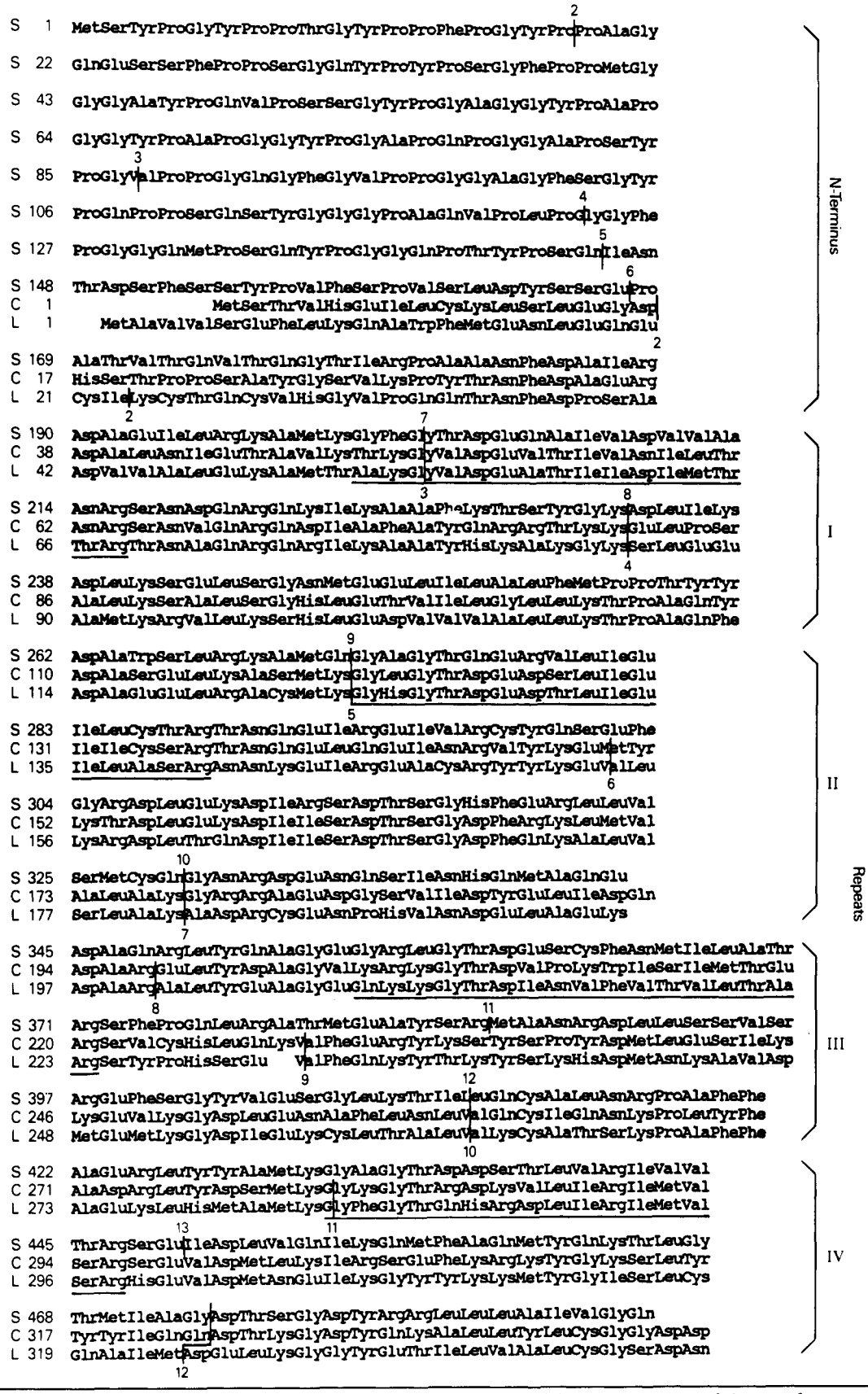
junction shared by both the calpactin I and lipocortin I genes (boundary 9). Boundary 12 in synexin does occur in the exact position and phase as splice junction 10 for both calpactin I and lipocortin I. However, within this repeat, both calpactin I and lipocortin I genes share splice junction 8, which is missing in the synexin gene. Finally, in the fourth repeat, the synexin gene completely deviates from the calpactin I and lipocortin I genes, in that the synexin splice junction, 13, occurs entirely isolated from the locations of the *two* exon/intron boundaries possessed in this repeat by the other two genes. In the latter cases, boundary 11 occurs in a common position and phase, while boundary 12 is offset by one residue, but preserves the phase. Thus, from an overall perspective, it appears that the gene structure within these three annexins is most conserved within the domain of the first repeat and that the similarities decrease in the second and third repeats and are extremely dissimilar in the fourth repeat. This is somewhat of a dilemma since the amino acid similarities among annexins are maximal in repeat 4 (Barton et al., 1991).

Nonetheless, we note that while the locations of the splice junctions vary, most splice junctions in the conserved C-terminal tetrad repeats correlate with the beginnings or endings of putative α-helical domains. These domains are defined by analogy with the crystal structure of endonexin II and annexin I (AnxV: Hüber et al., 1990a,b; AnxI: Weng et al., 1993). We have previously observed that allowed variations between human and mouse synexins also often occur at the beginnings or endings of putative α-helical domains (Zhang-Keck et al., 1993). However, we have yet to find any more specific correlation between the locations of splice junctions and amino acid substitutions.

Enhancer and Promoter Sequences in the Synexin Gene. Although widely distributed phylogenetically and in most mammalian tissues, AnxVII is generally of low abundance and appears to be among the least expressed of the mammalian annexins. It is therefore relevant to scrutinize the 5′ end of the anxVII gene for hints as to the possible genetic elements regulating expression (see Figure 5). Among those elements associated with promoter activity in other genes are two AP-1 transcription factor-binding sites located at –213 and –710 and one AP-2 transcription factor-binding site located 14 bp downstream from the 5′ end of the first intron. These binding sites can act as phorbol ester-inducible elements (Imagawa et al., 1987, Martin et al., 1988). Interestingly, the equivalent 5′ regions of pigeon *anx*I were found to contain one AP-1 site and one AP-2 site. Thus, *anx*I and *anx*VII genes could be stimulated by phorbol esters. The *anx*VII gene also contains two glucocorticoid responsive elements (GRE) at positions –1031 and –1040 upstream of the putative transcription initiation site. The presence of these elements is often indicative of genes that are positively regulated by steroid hormones (Overhauser & Fan, 1985) and may deserve some scrutiny in terms of the historical concept of AnxI as a mediator of steroid action.

The *anx*VII gene also contains a variety of known enhancers that positively activate transcription in a binding-site-dependent manner. These enhancers include GCN4 (Arndt & Fink, 1986), found twice at positions –204 and –689, ZESTE (Bourne, 1988), alone and overlapping once with a GAGA box (Biggin & Tjian, 1989), which occurs five times at –54, –282, –519, –1055, and +206 into the first intron. Adh 1 (Ferl & Nick, 1987) is present three times in the 5′ flanking

Table 3: Alignment of Amino Acids and Splice Junctions of Synexin, Calpactin I, and Lipocortin I[a]



[a] The unique N-terminal sequences for synexin (S), calpactin I (C), and lipocortin I (L) are shown on top of the sets of sequences for the four homologous repeats. Each repeat is labeled in the right margin, and in each repeat the endonexin fold motif is underlined. The amino acid numbering system for the different sequences is shown in the left margin. Splice junctions are demarcated by vertical lines. The placement of the line within the three-letter amino acid code indicates the phase of the exon/intron boundary. Exon numbers for calpactin I and lipocortin I are below the vertical lines, while those for synexin are above the vertical line. The AnxVII sequence contains the 22 amino acids encoded by the cassette exon inserted after position 145 in the protein.

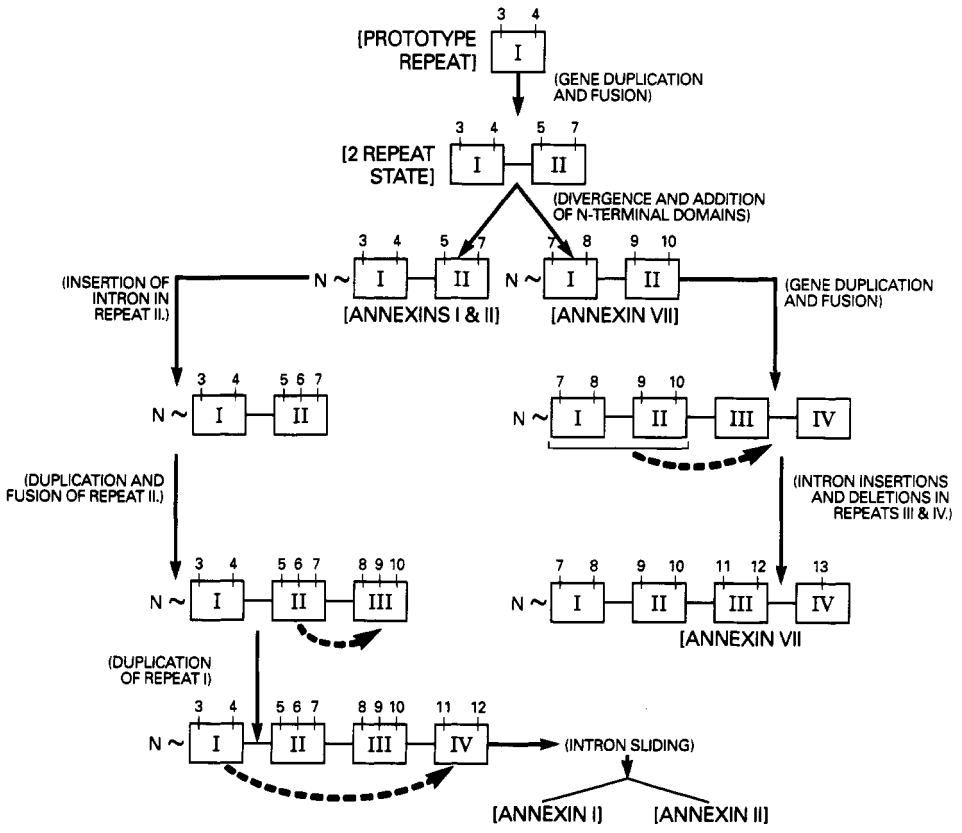


FIGURE 8: Model for the evolution of annexins, based on conserved and nonconserved splice junction locations in annexin genes. The prototype repeat (I) with two splice junctions (3 and 4) is shown at the top of the flow chart. Through the process of gene duplication and fusion (step 1), a two-repeat state is generated. At step 2, divergence occurs into pathways leading to annexins I and II (left pathway) and annexin VII (right pathway). In addition, the unique N-terminal domains are added. Along the synexin pathway, step 3 involves gene duplication and fusion to generate the tetrad repeat. The final state is generated by intron insertions and deletions in repeats III and IV (step 4). The pathway leading to annexins I and II is different and involves the insertion of an intron in repeat II (step 5), followed by duplication of repeat II and fusion to form repeat III (step 6). Duplication and fusion of repeat I then occurs (step 7), before annexins I and II are further distinguished by intron sliding (step 8). Other hypothetical pathways are possible, but in all cases the different splice junction patterns indicate that the two pathways are different.

region (–41, 699, and –994) and once at +170 in the first intron. Other well-studied consensus sequences in this region include JCV (Martin et al., 1985), CTF (Morgan et al., 1987), several Hsp70 sites (Wu et al., 1987), Lva (Spech and Baltimore, 1987), and a large number of dispersed CAP recognition sites (Snyder et al., 1984).

Some enhancer elements in the *anx*VII gene are known to be tissue-specific or regulated during development, differentiation, or stress. Inasmuch as a specific edited form of AnxVII occurs in brain, skeletal, and cardiac muscle, such elements could be of intrinsic interest. These include the mouse immunoglobulin heavy-chain enhancer, IgHC, at –741, the c-mos enhancer from the c-mos protooncogene at –717, two polyoma virus enhancer A-binding protein sites, PEA3, occurring at –213 and –802, and two whey acid promoter sites, WAP, which are activated during lactation, occurring at –534 and –858. Finally, a stress-related promoter site, CTF/CBP, is located at –572.

The mechanism by which most promoters function is not fully appreciated, and the list of potential promoter/enhancer sites in the *anx*VII gene is likely to be far from complete. However, the data in hand do provide clues as to which experimental situations might be expected to modify gene expression. To better understand the nature and significance of the 5′ region of synexin, more experimental work will be needed.

Chromosomal Assignment of the Human Synexin Gene. Human synexin is a single-copy gene, and its assignment to chromosome 10q21.1-21.2 clearly distinguishes synexin from other known human members of the annexin gene family (see Table 4). Calpactin I (*anx*II) has been reported to occur on four different chromosomes (Huebner et al., 1988; Spano et al., 1990). One locus, 4q21-31.1, corresponds to a locus also reported for endonexin II (*anx*V) (4q21-33.1; Tait et al., 1991; Modi et al., 1989; Hauptmann & Rentelingsperger, 1992) and close to the locus of *anx*III (4q13-22; Tait et al., 1991). A second *anx*II locus, 9qter-24, shows a close proximity to the reported locus of *anx*I, 9q11-22 (Huebner et al., 1988). A third reported locus for *anx*II is at 10q24, which is close to that reported here for synexin. The fourth locus for *anx*II is at 15q21-22 and shows no homologous location equivalent to any of the known annexins. Presently, it has not been determined whether the multiple *anx*II loci represent a multicopy gene, located at four independent sites, whether any of these sites represent pseudogenes of annexin II, or whether any of these sites have been inadvertantly identified

Table 4: Chromosomal Assignment of Annexin Genes

gene	chromosome	reference
annexin I	9q11-q22	Huebner et al., 1988
annexin II	4q21-q31.1 9pter-q34 10q-q24 15q21-q22	Huebner et al., 1988; Spano et al., 1990
annexin III	4q13-22	Tait et al., 1991
annexin V	4q21-33.1	Tait et al., 1991; Modi et al., 1989; Hauptmann & Rentlingsperger, 1992
annexin VI	5q32-q34	Davies et al., 1989
annexin VII	10q21.1-21.2	this work

because of the structural similarity of the different annexin genes.

Implications for the Mechanism of Evolution of Synexin and Other Annexins. On the basis of the distribution of splice junctions in the in the *anx*VII gene, we have deduced a relatively simple hypothetical mechanism for the evolutionary development of the conserved tetrad repeat structure (see Figure 8). As summarized for AnxVII in the right-hand branch of the flow chart in Figure 8, the prototype repeat, bearing two splice junctions, becomes a diad through gene duplication and fusion. The N-terminal domain is added, and through a second round of gene duplication and fusion, the tetrad repeat is formed. Finally, through a process of intron insertions and deletions in repeat IV, the mature structure is generated bearing two splice junctions in repeats I–III and only one splice junction in repeat IV.

Assuming that the entire annexin gene family may have evolved in a similar fashion from a common prototype repeat I, we can now direct our attention to the hypothetical evolutionary pathway for annexins I and II. However, as shown in the left-hand branch of the flow chart in Figure 8, the distribution of splice junctions indicates that the simplest pathway is clearly divergent from that of AnxVII. Briefly, through gene duplication and fusion, the prototype annexin domain with two introns (junctions 3 and 4) becomes doubled to form a structure with junctions 3–7. An intron is then inserted in repeat 2 (junction 6). Repeat 2 is duplicated to form a three-repeat protein in which junctions 5–7 become junctions 8–10, respectively, in repeat 3. The fourth repeat is then formed by a duplication of repeat 1, in which junctions 3 and 4 become junctions 11 and 12, respectively, and the modified domain is then inserted into the 3′ end of the gene. Presumably an identical model is obtained for *anx*III, since the splice junctions are virtually identical to those for *anx*I and -II (Tait et al., 1993).

Alternative models for the evolution of *anx*I and -II have been proposed (Kovacic et al., 1991; Horlick et al., 1991), and Kovacic et al., (1991) have specifically based their model on repeat IV as the primitive repeat. However, if we were to have chosen repeat IV as the prototype repeat to analyze AnxVII, matters would be profoundly more complicated than they already are. The reasons are that repeat IV in *anx*VII has one splice junction, while repeats I–III have two each. Thus, while different repeats could be chosen, with good reason, as the protype, one would still be left with the conclusion that divergent mechanisms would separate *anx*VII from *anx*I and -II. The sequence of the mouse synexin cDNA is highly similar to that of human synexin (Zhang-Keck Burns et al., 1993), and analysis of the mouse synexin gene, now in progress, will allow us to verify whether the apparent divergence of the human synexin gene from the other two annexins is general.

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