

The hinge region of chicken annexin I contains no site for tyrosine phosphorylation

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Annexin I (AnxI) is a calcium-dependent membrane binding protein which has been implicated in various physiological activities. The region of the chicken *anxI* cDNA encoding the first 130 amino terminal residues was cloned by reverse transcription PCR in order to determine the relationship of its variable amino-terminal regulatory region with other known annexins. This nucleotide sequence shows 86% identity with pigeon AnxI isoforms, and 57% with its human homolog. The protein encoded by the chicken *anxI* cDNA lacks the canonical epidermal growth factor receptor/kinase phosphorylation site, which is present in AnxI of other species. In contrast, the putative protein kinase C phosphorylation site of the amino-terminus is present in the chicken AnxI. Whereas the pigeon genome contains two *anxI* genes, genomic Southern analysis shows that in the chicken AnxI is encoded by only a single gene. These data suggest that AnxI has undergone significant sequence variation in the avians, and clarifies the relationships of the avian *anxI* genes with their ancestral homologs.

Annexin I; Lipocortin I; Tyrosine kinases; Protein kinase C; Molecular evolution; Chicken

1. INTRODUCTION

Annexin I (AnxI; also referred to as lipocortin I) is a member of a class of homologous proteins that bind to membrane phospholipids in a calcium-dependent manner. There are at least 12 distinct members in this group, which are widely distributed across both species and tissues and have been implicated in diverse physiological activities (see [1–3] for reviews). While both the Ca^{2+} and the membrane binding activities of the annexins reside in the core domain, the amino-terminal tail plays a role in the regulation of these proteins [4–8].

AnxI is one of the best characterized members of the group [9,10]. While mammals have only one *anxI* gene encoding a single protein product, the pigeon genome contains two *anxI*-type genes. The first to be discovered, *cp35*, is a major prolactin-induced gene in the cropsac. Its protein product is tissue-specific and lacks the typical AnxI phosphorylation sites in its leader domain [11]. The second isoform, referred to as *cp37*, encodes a protein which is constitutively expressed, and contains AnxI consensus phosphorylation sites [12]. The two pigeon isoforms share 93% amino acid identity, but differ con-

siderably in the hinge region, which is encoded by the first half of exon three [10]. Based upon these findings it was speculated that the phosphorylation sites of *cp37* evolved independently in the Aves.

We now report the cDNA sequence of a large amino-terminal portion of chicken AnxI and present evidence that this gene exists as a single copy in the chicken. The chicken sequence clarifies the evolution of *anxI* in birds, and provides new insights into the regulation of its protein.

2. EXPERIMENTAL

2.1. Northern blot assay of annexin I mRNA

Total RNA from the cropsac of prolactin treated (200 $\mu\text{g/day}$ for 3 days) pigeons and from liver, lung and cropsacs of 1- or 3-day-old chickens, were extracted by RNAzol B (Cinna/Biotech, Houston, TX, USA) and electrophoresed (15 $\mu\text{g/lane}$) through 1% agarose, 1.1 M formaldehyde gels containing 0.5 $\mu\text{g/ml}$ ethidium bromide. After electrophoresis the samples were transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, NH) by capillary blotting and probed by standard Northern blot technique [12].

2.2. Genomic DNA extraction and Southern analysis

Chicken liver tissue fragments (~0.5 g) were ground in liquid nitrogen and digested in 6 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, 20 $\mu\text{g/ml}$ RNAase and 0.1 mg/ml proteinase K) at 50°C for 16 h. The DNA was then extracted 3 times with an equal volume of phenol/chloroform/isoamyl alcohol, precipitated with 1/2 vol. of 7.5 M ammonium acetate and 2 vol. of 95% ethanol and recovered as described [13]. Genomic DNA aliquots were restriction digested, ethanol precipitated, and fractionated (20 $\mu\text{g/lane}$) on 0.8% agarose gel. The DNA was then transferred to a nylon membrane, fixed and hybridized [14]. For reprobing, the blots were stripped by boiling for 20 min in 0.1 × SSC and 0.5% SDS.

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Abbreviations EGF, epidermal growth factor; PKC, protein kinase C; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; AnxI, annexin I.

2.3. Oligonucleotide primers

Oligonucleotides used in polymerase chain reaction (PCR) were synthesized according to previously reported *AnxI* sequences of sponge, human and pigeon ([15,16] and [11] and [12], respectively). The sense primers (5'-CGCGCATGCATGGCTATGGTATCAGA-ATTTC-3') corresponded to the characteristic conservative segment at the beginning of *AnxI* type sequences and designed to contain a *SphI* restriction site (underlined) and CGC clamp at the 5' end. The anti-sense primer (3'-CCCGAACCTTGACTACTTCTACTCGAG-CGC-5') contained a *SacI* site (underlined) and corresponded to a conservative sequence located at the end of exon 5 and the beginning of exon 6 (see the primer positions in Fig. 3).

2.4. PCR amplification of chicken *AnxI* cDNA

Single-stranded cDNA was synthesized in a 20 μ l reaction containing: 1 \times Taq DNA Polymerase buffer, 1.25 mM $MgCl_2$, 0.1–0.2 μ g total RNA, 5 pmol antisense primer, 20 U of RNasin ribonuclease inhibitor (Promega), 1 mM of each deoxynucleotide triphosphate (dNTP) and 7.5 U of AMV Reverse Transcriptase (Promega). The mixture was incubated at 42°C for 1 h and the reaction terminated by heating at 95°C for 5 min. The reverse transcription reaction was then diluted to a final volume of 100 μ l in 1 \times Taq DNA polymerase buffer or Pfu DNA polymerase buffer, 5 μ M of each of the sense and antisense primers and 5 U of either Taq or Pfu DNA polymerase. Amplification was performed at a temperature profile of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, for 38 cycles. The right size product was extracted from a 1% agarose gel using a Prep-A-Gene DNA purification kit (Bio-Rad Lab. Richmond, CA). Reamplification reactions were carried out by the same procedure as the above amplification reaction using 1 ng of the purified cDNA fragment as a template.

2.5. Cloning and sequencing the PCR cDNA fragment

The double-strand PCR products were digested with *PstI* and *SacI*, purified and ligated into cleaved and dephosphorylated pGEM3 plasmids (Promega). Competent cells (DH5a, BRL) were transformed and the positive colonies were selected by ampicillin resistance. Five independently cloned PCR products were sequenced on both strains, by the dideoxy chain termination method using Sequenase (US Biochemical, Cleveland, OH).

2.6. Sequence alignment

The degree of identity between chicken nucleotides and amino acid sequences and other *AnxI* relatives, was calculated for the various structural domains of the protein, from optimal pairwise alignments. For these calculations the sequences defined by the PCR primers were ignored. This includes: the Leader Domain – residues 8–20; the Hinge Region – residues 21–41 (human *AnxI* numbering); and residues 42–128 of the Core Domain. The alignments were obtained independently for each pair, by CLUSTAL V software [17].

3. RESULTS AND DISCUSSION

Northern blot analysis of chicken lung, liver and cropsac revealed high level expression of a 1.4 kb mRNA in the cropsac, but not in liver or lung (Fig. 1). Cropsac RNA was used for reverse transcription PCR (rtPCR) of the desired *AnxI* fragment. The 410 bp PCR product, proven by sequencing to be *AnxI* (see below), was used as a probe in genomic Southern blot analysis (Fig. 2A). The various restriction enzymes generated 1–5 hybridizing bands which ranged between 0.5–8 kb. This contrasts with *anxI* in pigeon genome, which yielded a much more complex Southern hybridization pattern [14]. The hybridization patterns of chicken *anxI* and *vimentin* (Fig. 2B) were of similar complexity. Vi-

mentin is encoded by a single gene in the chicken (9 exons, 8.5 kb; [18]). These data indicate that chicken *anxI*, unlike pigeon, is a single-copy gene.

Chicken *anxI* cDNA, synthesized independently by both Taq and Pfu DNA polymerases, was cloned and sequenced. The resulting 390 base sequence obtained from 5 independent clones, is presented in Fig. 3, along with its deduced polypeptide of 130 amino acids. The 130 amino acid region that we cloned for the chicken corresponded to the entire amino-terminal regulatory domain, all of repeat 1 and the first helix of repeat 2. This fragment was chosen because the intent was primarily to analyze the variable amino-terminus, not to repeat the cloning of the conserved core domain. As would be expected in pairwise alignments of the 130 amino acid region, the chicken polypeptide sequence shared higher identity with both pigeon relatives (80% on average) than with either human (72%) or sponge (55%).

It is well established that Tyr-21 of the hinge region of mammalian *AnxI* is a high affinity substrate for the EGF receptor/kinase [19,20]. Phosphorylation of Try-21 renders *AnxI* 20-fold more sensitive to proteolysis at Lys-26 [20]. The hinge region also contains substrate sites for PKC at Ser-27 and Thr-24 [7]. The chicken *AnxI* like pigeon cp35, has a cysteine in position 21, instead of the tyrosine contained by all other relatives (Figs. 3 and 5). With cp37 it shares the Ile-Lys-Ser PKC

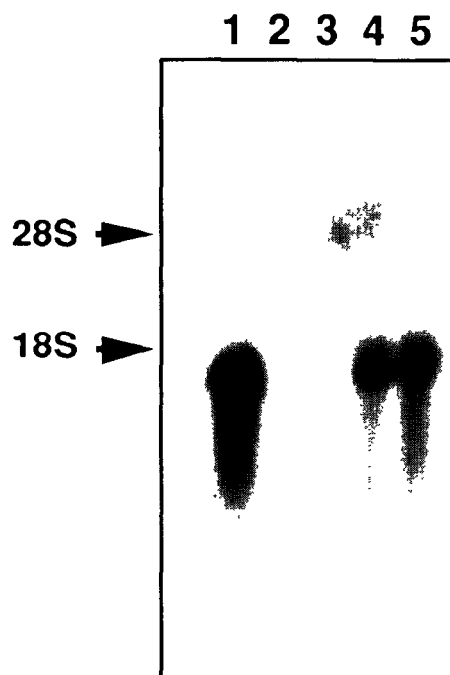


Fig. 1. Expression of *AnxI* in chicken tissues. Total RNA from prolactin-stimulated pigeon cropsac (lane 1 = positive control) and from 1-day-old chicken liver (lane 2), lung (lane 3) and cropsac (lanes 4 and 5) were separated through a 1% formaldehyde-containing agarose. The RNA was then capillary transferred to a nylon membrane and probed with nick-translated cp35 insert cDNA.

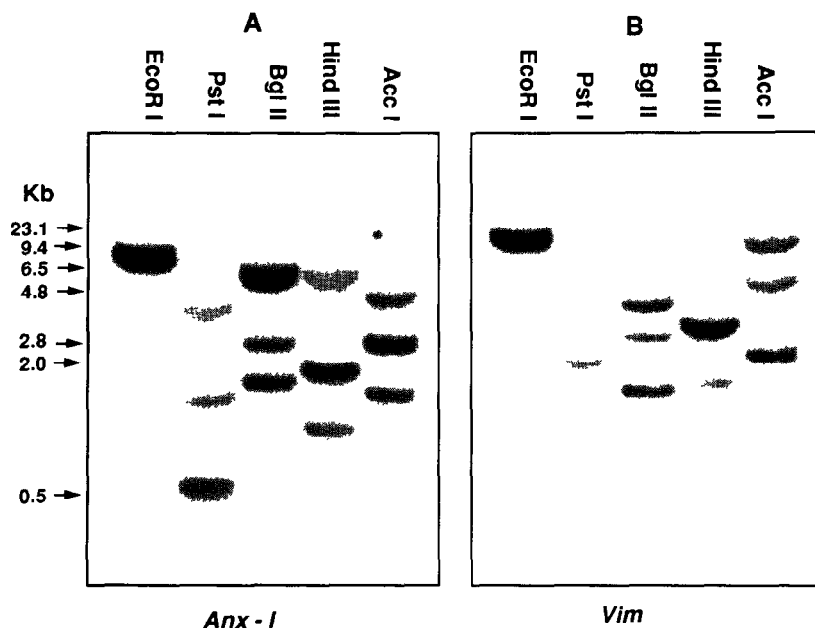


Fig. 2. Genomic Southern analysis of chicken AnxI. High molecular weight chicken liver DNA was digested with restriction enzymes (as noted above the figure) and fractionated (20 μ g/lane) on 0.8% agarose gel. Following capillary transfer the blot was probed with chAnxI cDNA fragment (A), stripped and reprobed with nick-translated pigeon vimentin cDNA insert (B).

phosphorylation site at positions 22–24, and the Lys-Gly-Gly motif at positions 26–28 (Figs. 3 and 5). The following 6 amino acids (29–34) show almost no identity with either pigeon isoform nor with nonavian relatives (see alignment in Fig. 5).

A multiple alignment of the hinge region nucleotide

sequences from all species studied to date is shown in Fig. 4. Gaps in the alignment of avian AnxI and other species were located at two separate locations: positions 77–84 (numbered from the initiation codon) and 98–100. Except for a short sequence of 8 nucleotides (positions 104–111), most of the hinge region showed a re-

ATG	GCT	ATG	GTA	TCA	GAA	TTT	CTG	AAG	CAG	GCA	TGG	TTC	ATG	GAC	AAC	CAG	51
Met	Ala	Met	Val	Ser	Glu	Phe	Thr	Lys	Gln	Ala	Trp	Phe	Met	Asp	Asn	Gln	17
GAG	CAG	GAA	TGT	ATT	AAA	AGT	TCA	AAA	GGT	GGC	TCT	TCA	GTA	CAG	TCA	CGC	102
Glu	Gln	Glu	Cys	Ile	Lys	Ser	Ser	Lys	Gly	Gly	Ser	Ser	Val	Gln	Ser	Arg	34
CCA	AAC	TTT	GAT	CCC	TCA	GCT	GAT	GTT	TCT	GCT	TTG	GAT	AAA	GCT	ATT	ACT	153
Pro	Asn	Phe	Asp	Pro	Ser	Ala	Asp	Val	Ser	Ala	Leu	Asp	Lys	Ala	Ile	Thr	51
GTA	AAG	GGT	GTA	GAT	GAA	GCC	ACC	ATC	ATT	GAC	ATC	TTG	ACT	AAA	AGA	ACA	204
Val	Lys	Gly	Val	Asp	Glu	Ala	Thr	Ile	Ile	Asp	Ile	Leu	Thr	Lys	Arg	Thr	68
AAT	GCT	CAA	CGT	CAG	CAG	ATC	AAA	GCT	GCC	TAT	CAA	CAG	GCA	AAA	GGA	AAG	255
Asn	Ala	Gln	Arg	Gln	Gln	Ile	Lys	Ala	Ala	Tyr	Gln	Gln	Ala	Lys	Gly	Lys	85
AGC	CTG	GAA	GAA	GAC	TTG	AAA	AAG	GTG	CTG	AAA	AGC	CAC	TTG	GAA	GAT	GTT	306
Ser	Thr	Glu	Glu	Asp	Leu	Lys	Lys	Val	Thr	Lys	Ser	His	Leu	Glu	Asp	Val	102
GTT	GTT	GCT	CTT	CTC	AAA	ACT	CCA	GCT	CAG	TTT	GAT	GCT	GAA	GAA	TTA	AGG	357
Val	Val	Ala	Thr	Thr	Lys	Thr	Pro	Ala	Gln	Phe	Asp	Ala	Glu	Glu	Leu	Arg	119
GCC	TCT	ATG	AAG	GGG	CTT	CGA	ACT	GAT	AGA	AGA							390
Ala	Ser	Met	Lys	Gly	Thr	Gly	Thr	Asp	Arg	Arg							130

Fig. 3. Nucleotide and deduced amino acid sequences of chAnxI cDNA fragment. Chicken cropsac total RNA (0.1 μ g) was reverse transcribed and amplified by PCR using primers which are specific for AnxI (underlined). The PCR products were cloned and sequenced as described.

	60	70	80	90	100
sAnxI	AGAACAAGAA	TATATTGAAA	TCGTAAAATC	CTACAAAGGT	GGTCCTGCTC
mAnxI	AGAACAAGAA	TATGTTCAAG	CTGTAAAATC	ATACAAAGGT	GGTCCTGGGT
rAnxI	AGAGCAGGAA	TATGTTCAAG	CTGTAAAATC	CTACAAAGGT	GGTCCTGGAT
gpAnxI	AGAACAAGAC	TATGTCAAAA	CTGTGAAGTC	ATCCAAAGGT	GGTCCTGGCT
hAnxI	AGAGCAGGAA	TATGTTCAAA	CTGTGAAGTC	ATCCAAAGGT	GGTCCCGGAT
chAnxI	GGAGCAGGAA	TGTATTAAAA	GTTCA---	-----AAAGGT	GGCTCTT---
cp37	GGAAACAGGAA	TATATTAAAA	GTGTA---	-----AAAGGA	GGCCCTG---
cp35	<u>GGAGCAGGAA</u>	<u>TGTATTAAAT</u>	<u>GTACA---</u>	<u>-----CAATGT</u>	<u>GTCCATG---</u>
	*****	*****	++++	*****	**++++
	110	120	130	140	150
sAnxI	ATGCAGTGAG	CCCATACCCC	TCCTTCGATC	CGTCCTCGGA	TGTTGCTGCT
mAnxI	CAGCAGTGAG	CCCCTACCCCT	TCCTTCAATG	TATCCTCGGA	TGTTGCTGCC
rAnxI	CAGCAGTGAG	CCCCTACCCCT	TCCTTCAATC	CGTCCTCGGA	TGTTGCTGCC
gpAnxI	CAGCCGTGAG	CCCCTACCCC	ACCTTCGATC	CTTCCTCGGA	CGTTGCTGCT
hAnxI	CAGCGGTGAG	CCCCTATCCT	ACCTTCAATC	CATCCTCGGA	TGTCGCTGCC
chAnxI	CAGTAC---A	GTCACGCCCA	AACTTTGATC	CCTCAGCTGA	TGTTTCTGCT
cp37	TAGTACCACA	ACAACAGCCT	AACTTTGATC	CATCAGCTGA	TGTTGTTGCT
cp35	<u>GAGTACACACA</u>	<u>ACA---GACT</u>	<u>AACTTTGATC</u>	<u>CATCAGCTGA</u>	<u>TGTTGTTGCT</u>
	+++	+++	+++	+++	*****

Fig. 4. Multiple alignment of nucleotide sequences of the hinge region (indicated by the upper line) of AnxI from sponge (sAnxI; [15]), mouse (mAnxI; [21]), rat (rAnxI; [22]), guinea pig (gpAnxI; [23]), human (hAnxI; [16]), chicken (chAnxI; this paper) and pigeon (cp35 and cp37; [11] and [12], respectively). Gaps were automatically introduced by CLUSTAL V software. The asterisk and plus symbols on the bottom of the sequence indicate similarity between avian and nonavian sequences. Asterisks represent positions that are identical in all sequences, plusses indicate positions in which the same base appears in at least one of the avian and one of the nonavian sequences. Identical bases shared by chicken and at least one of the pigeon isoforms are underlined.

	Leader Domain	EGFR ↓	PKC ↓	"Hinge"	
sAnxI	MAMVSEFINQ	ACYLEKQEQE	YIEI'KS'KG	GFPH'EFIP	SFDPS 45
mAnxI	MAMVSEFLKQ	ARFLENQEQE	Y'K'KS'KG	GFPH'EFIP	SFNVS
rAnxI	MAMVSEFLKQ	ACYIEKQEQE	Y'K'KS'KG	GFPH'EFIP	SFNPS
gpAnxI	MSMVSEFLKQ	AYFIDNQEQD	Y'K'KS'KG	GFPH'EFIP	SFDAS
hAnxI	MAMVSEFLKQ	AWFIEHEEQE	Y'K'KS'KG	GFPH'EFIP	TFNPS
chAnxI	MAMVSEFLKQ	AWFMDNQEQE	CIK3'---KG	GFPH'EFIP	NFDPS
cp37	MAMVSEFLKQ	AWFMEHQEQE	YIKS'---KG	GFPH'EFIP	NFDPS
cp35	MAVVSEFLKQ	AWFMENLEQE	CIK1'---QC	VH-GVPQ-QT	NFDPS
	*	*****	* ** *		** *
	Repeat I				
sAnxI	SDVAA	LHKAIMVKG	DEATILDLLT	KRYNAQRHHL	KAVYIQETGE 90
mAnxI	SDVAA	LHKAIMVKG	DEATIIDLIT	KRTNAQRQQI	KAAYLQENGK
rAnxI	SDVAA	LHKAIMVKG	DEATIIDLIT	KRTNAQRQQI	KAAYLQETGF
gpAnxI	SDVAA	LHKAITVKG	DEATIIDLIT	KRNNAQRQQI	KAAYLQEKGE
hAnxI	SDVAA	LHKAIMVKG	DEATIIDLIT	KRNNAQRQQI	KAAYLQETGF
chAnxI	ADVVA	LHKAITVKG	DEATIIDLIT	KRTNAQRQQI	KAAYQKAKGK
cp37	ADVVA	LDKAMTAGV	DEATIIDIMT	KRTNAQRQQI	KAAYQKAKGK
cp35	ADVVA	LEKAMTAGV	DEATIIDIMT	TPTNAQRQQI	KAAYHKAKGK
	** *	*****	*****	*****	** *
	Repeat II				
sAnxI	PLDETLKKAL	TGHIEQLLLA	MIKAPAQFDG	NELRAAMKAV	GTDBE 135
mAnxI	PLDETLKKAL	TGHLEEVVLA	MLKTPAQFDA	DELRGAMKGL	GTDED
rAnxI	PLDETLKKAL	TGHLEEVVLA	MLKTPAQFDA	DELRAAMKGL	GTDED
gpAnxI	PLDEALKKAL	TGHLEEVVLA	LLKTPAQLDA	DELRAAMKGL	GTDED
hAnxI	PLDETLKKAL	TGHLEEVVLA	LLKTPAQFDA	DELRAAMKGL	GTDED
chAnxI	SLEEDLFFVL	KSHLEDVVVA	LLKTPAQFDA	EELRASMKGL	GTDED
cp37	SLEEAMKFFVL	KSHLEDVVVA	LLKTPAQFDA	EELPACMKGL	GTDED
cp35	SLEEAMKFFVL	KSHLEDVVVA	LLKTPAQFDA	EELRACMKGH	GTDED
	****	*****	*****	*****	*****

Fig. 5. Comparison of the amino acid sequences of the leader domain (residues 1–20) the hinge region (residues 21–41) and the first two repeats of the core domain (residue 43–135) of AnxI. Species are as listed in Fig. 4. Gaps were inserted according to the nucleotide sequence alignment rather than by independent alignment of the amino acid sequence. Identical and conservative substitutions across the sequences are indicated by asterisks. The arrows denote the phosphorylation sites for PKC and for the EGF receptor/kinase in mammals. Motifs which are conserved between avians (excluding cp35) and nonavians are shadowed. A segment that is highly diverse within the avians is boxed.

markably high similarity between avian and nonavian sequences (Fig 4). Therefore, it seems reasonable to conclude that in spite of the low amino acid identity, the avian hinge region is homologous to the nonavian one. Its diversity then, is the result of deletion events and base substitutions, rather than an insertion of a new segment of DNA [12].

The amino acid sequence of the chicken AnxI fragment and other homologues are compared in Fig. 5. In the leader domain and the first repeat segments the avian sequences showed a great deal of similarity with the nonavian, with most of the substitutions being conservative. While the selective pressure acting to conserve the structure of the core domain is evident, the reason for strong conservation in the leader domain is still to be discovered. In the hinge region the similarity between avians and nonavians was found to be very low (Fig. 5). In conformity with the nucleotide alignments (Fig. 4) 3- and 1-residue gaps were placed at positions 26 and 33, respectively. An alternative alignment in which all missing residues were grouped together in a single gap at positions 35 for cp35, and 36 for cp37 and chAnxI, was obtained by CLUSTAL V [12]. However, the two-gap alignment, consistent with the nucleotide analysis, was preferred since it offers better interpretations of the homologous relationships between sequences. The alignment in Fig. 5 suggests that the Lys-Ser, putative PKC phosphorylation site, shared by chAnxI and cp37, are encoded by codons which are not homologous with those encoding the analogous residues in nonavians. Furthermore, Lys-26 (per human numbering), which is a protease-sensitive site, is also not homologous between the two groups. It is of interest to note that in the avian sequences, Lys-29 (per human numbering) together with the successive Gly-Gly residues are homologous with identical sequences, in other species. Another feature of avian AnxI is a highly variable segment corresponding to Ser-29 through Arg-34 of chAnxI. At the polypeptide sequence level the changes outlined here appear complex, but they require a minimal number of nucleotide changes (Fig. 4).

Based on the above observations one can propose that the hinge region of avian *anxI* genes arose by modifications of the ancestral gene sequences that preserved some of the potential regulatory mechanisms, but eliminated others. The presence of the two AnxI genes in columbids is a special case. Our model suggests that the PKC Ser-24 site is the major regulation site in AnxI, and

that Tyr-21 plays a subsidiary role. The polymorphism of AnxI genes in the two avian species examined to date argues that an extensive analysis of avian AnxI will yield important molecular and evolutionary insights.

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