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Correlation of Gene and Protein Structure of Rat and Human Lipocortin I†

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ABSTRACT: Lipocortins (annexins) are a family of calcium-dependent phospholipid-binding proteins with phospholipase A_2 inhibitory activity. The characteristic primary structure of members of this family consists of a core structure of four or eight repeated domains, which have been implicated in calcium-dependent phospholipid binding. In two lipocortins (I and II) a short amino-terminal sequence distinct from the core structure has potential regulatory functions which are dependent on its phosphorylation state. We have isolated the rat and the human lipocortin I genes and found that they both consist of 13 exons with a striking conservation of their exon–intron structure and their promoter and amino acid sequences. Both lipocortin I genes are at least 19 kbp in length with exons ranging from 57 to 123 bp interrupted by introns as large as 5 kbp. Each of the four repeat units of lipocortin I are encoded by two consecutive exons while individual exons code for the highly conserved putative calcium-binding domains. The promoter sequences in the rat and in human genes are highly conserved and contain nucleotide sequences characterized as enhancer sequences in other genes. The structure of the lipocortin I gene lends support to the hypothesis that the lipocortin genes arose by a duplication of a single domain.

Lipocortins (lately also referred to as annexins) are a group of calcium/phospholipid-binding proteins with phospholipase A_2 (PLA_2) inhibitory activity. Originally characterized as dexamethasone-induced inhibitors of eicosanoid production [for review, see Flower (1985, 1988)], members of the lipocortin family are now implicated in the regulation of a variety of biological events, such as blood coagulation and differentiation, and in various aspects of the immune response and inflammation (Maurer-Fogy et al., 1988; Funakoshi et al., 1987; Cirino & Flower, 1987a,b; Cirino et al., 1989; Ishizaka, 1985).

Lipocortin-like proteins have also been isolated by other groups on the basis of their calcium-dependent phospholipid-binding properties and have been given other names, including p35 (Fava & Cohen, 1984), p36 (Gerke & Weber, 1985), calpactins (Glenney, 1986), endonexins and p68 (Davies & Crumpton, 1985; Gerke & Weber, 1984), annexins (Geisow et al., 1987), calcimedins (Mathew et al., 1986), calelectrins (Geisow, 1986a), chromobindins (Creutz et al., 1987), and proteins I–III (Weber et al., 1987; Shadle et al., 1985; Burns et al., 1989) [for reviews, see Klee (1988), Flower (1988), and Wallner (1989)]. Eight distinct members of the mammalian lipocortin family have now been identified (Pepinsky et al., 1988; Haigler et al., 1989; Hauptman et al., 1989). The amino acid sequences of the lipocortins are highly conserved between species with all members showing 40–50% sequence homology to each other.

Lipocortins I and II appear to play a role in signal transduction: lipocortin I in EGF-dependent cellular proliferation and lipocortin II in oncogenic transformation. Lipocortin I (p35, calpactin II) is the major physiologic substrate of the EGF-induced EGF receptor kinase (Pepinsky & Sinclair, 1986; Sawyer & Cohen, 1985), and lipocortin II (p36, calpactin I) is identical with the major substrate of viral and growth factor dependent protein kinases (Huang et al., 1986; Erikson & Erikson, 1980; Saris et al., 1986).

The characteristic structure of lipocortin-like proteins consists of a 4-fold repeated domain of 70 amino acids (Wallner et al., 1986; Kretsinger & Creutz, 1986; Geisow, 1986b; Weber & Johnsson, 1986). Lipocortin I interacts with cellular membranes and vesicles in a calcium-dependent manner. A highly conserved region of 14 amino acids within each repeat unit has been implicated as the calcium-binding site (Weber & Johnsson, 1986; Schlaepfer & Haigler, 1987). The structure of this putative calcium-binding site is different from the EF-hand structure of other calcium-binding proteins.

† The genetic sequence in this paper has been submitted to GenBank under Accession Number J05339.
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A second conserved sequence in each domain of lipocortin I may represent the site of phospholipid binding (Geisow, 1986b).

The amino-terminal sequences of lipocortins are distinct from the repeated core structure and show little homology to each other (Pepinsky et al., 1988). In lipocortins I and II these sequences contain sites for tyrosine and serine/threonine phosphorylation. The state of phosphorylation is known to influence the calcium/phospholipid-binding affinity of the core structure (Schlaepfer & Haigler, 1987; Ando et al., 1989) and the heterotetramer formation of lipocortin II with the p10 subunits (calpactin I, protein I) (Johnsson et al., 1986a,b; Glenney & Tak, 1985). Hence, a regulatory role has been suggested for these N-terminal sequences.

Human lipocortin I is coded for by a single gene, located on chromosome 9 and mapping to the region 9q11–9q22 (Wallner et al., 1986; Huebner et al., 1988). One of the four genes mapped for human lipocortin II is also located on chromosome 9 (9pter–q134 proximal to *abl*), possibly close to the lipocortin I gene (Huebner et al., 1988).

We report here the isolation and the characterization of the rat and human lipocortin I genes. Both genes are highly conserved in their intron–exon structure and their promoter regions, as well as in their coding sequences. The structural organization of the lipocortin I gene strongly suggests that this gene has arisen through a duplication of a functional domain.

Materials and Methods

Isolation of Rat and Human Genomic Clones. Two rat genomic libraries (gifts of Dr. T. Sargent, NIH) constructed from either *Eco*RI partially digested or *Hae*III partially digested Sprague-Dawley liver DNA in Charon 4A arms (Sargent et al., 1979; Yu-Lee et al., 1986) were screened with a combination of oligonucleotide probes essentially as described (Woo, 1979). Briefly, a set of degenerate antisense oligonucleotide probes were synthesized on the basis of the amino acid sequences of rat lipocortin I peptides (Wallner et al., 1986). The probes were 5′ end labeled with [γ-^{32}P]ATP (Maxam & Gilbert, 1980), and their degeneracy was reduced by Northern blot analysis using rat lung RNA as described previously (Wallner et al., 1986). *Eco*RI fragments of positive phage λRLE1-4 were subcloned into pUC8 (Vieira & Messing, 1982) and overlapping *Eco*RI and *Hin*dIII fragments of λRLH14-1 subcloned into pUC19 and characterized by partial sequence determination.

Phage λ28p11 was isolated from a partial *Sau*3A human fibroblast genomic library (EMBL/5X, a gift of M. Pasek, Biogen Inc.) by using oligonucleotide probes to the 5′ untranslated sequence of the human lipocortin I cDNA. Phage λ43 was isolated by screening a partial *Hae*III/*Alu*I human liver genomic library (Lawn et al., 1978) with a 1-kb *Eco*RI fragment of plasmid pLiptrc155A (Wallner et al., 1986). Positive phages were further characterized by subcloning of overlapping restriction fragments and by sequence determination of these subclones.

Mapping and Sequencing of the Rat and Human Genes. The insert of phage λRLH14-1 was mapped by constructing overlapping *Eco*RI (pEE5, pEG13, pEF9, pEG16) and *Hin*dIII (pHC22, pHD26, pHE35, pHH18, pHE32) subclones. The overlapping sequences were located by Southern blot analysis using either restriction enzyme fragments or oligonucleotides specific to each subclone as hybridization probes. Only subclone pEG13 was fully sequenced by Maxam and Gilbert sequencing (Maxam & Gilbert, 1980). Subclone pHD26 was sequenced only partially in order to locate exon 1 and to determine the immediate 3′ intron sequence. All other subclones were characterized by restriction mapping and Southern blot analysis. The insert of λRLE1-4 was digested with restriction enzyme *Eco*RI, and isolated fragments were subcloned into pUC8 (pLipoE10, pLipoD1, pLipoF2, pLipoC1). Their locations in the λRLE1-4 insert were determined by Southern blot analysis with human cDNA specific oligonucleotide probes. Subclones pHH18 and pHE32 were used as hybridization probes in a Southern blot analysis to establish the extent of overlap between λRLH14-1 and λRLE1-4. Oligonucleotide probes specific to the human lipocortin I cDNA were used to sequence all rat lipocortin exons by the method outlined below and to establish the intron–exon boundaries in the rat genomic clones. The sequences of some of the introns were determined by sequencing only one strand of DNA. However, all intron–exon junctions were carefully sequenced from both strands (approximately 20 nucleotides each).

For the human gene, exons 3, 4, and 5 were subcloned as a 4.2-kb *Eco*RI fragment from clone λ43 and characterized by DNA sequencing (Maxam & Gilbert, 1980). All other human exon boundaries were determined by an adaptation of the genomic sequencing technique of Church and Gilbert (1984) by sequencing leftward and rightward from the site of hybridization of exon-specific oligomer probes. For example, a probe of sequence AAAGTCCTCAGATCGGTCAC hybridizes to the right of a *Bst*E2 site at nucleotide 630 in the human cDNA (Wallner et al., 1986). Maxam and Gilbert chemical cleavages on *Bst*E2-digested DNA, when electrophoresed, electrotransferred to a nylon membrane, and hybridized with a ^{32}P-labeled probe, provide sequence data adjacent to the probe rightward, thus directly providing the exon–intron boundary without subcloning.

Primer Extension. To determine the transcriptional sites, rat and human poly(A) RNA were prepared as described (Chirgwin et al., 1979), and primer extension was performed essentially as described (Wallner et al., 1986). Oligonucleotides homologous to the 5′ sequence of rat or human lipocortin I cDNA were used as primers.

Results

Cloning and Characterization of the Rat and Human Lipocortin I Genes. The purification and peptide sequencing of rat lipocortin I, the synthesis of oligonucleotide probes based on this sequence, and the isolation of the human lipocortin I cDNA have been described previously (Wallner et al., 1986; Pepinsky et al., 1986). The degeneracy of the oligonucleotide probes was decreased by hybridization to rat RNA in a Northern blot analysis as described (Wallner et al., 1986). One oligonucleotide subpool, Lipo2 (64-fold degenerate), hybridized to an 1800-nucleotide transcript in rat lung RNA (data not shown) and was used to screen a rat genomic library. The insert from a positive clone, λRLE1-4, was analyzed by restriction mapping; overlapping restriction fragments were subcloned, and their location in the rat gene was assigned by hybridization to discrete human lipocortin I cDNA restriction fragments. A summary of this mapping analysis is shown in Figure 1. DNA sequencing of these subclones revealed that the 15-kb insert of phage λRLE1-4 contained the complete rat lipocortin I coding sequence, but was missing the 5′ untranslated region. To obtain this sequence, the 5′ end of the rat lipocortin I mRNA was determined by primer extension analysis. This sequence information was then used to design an oligonucleotide probe which was used to isolate a phage, λRLH14-1, that contained the promoter and flanking sequences including exon 1. Exon 1 and exon 2 are separated by a 5.5-kb intron (Figure 1). Mapping and sequencing of

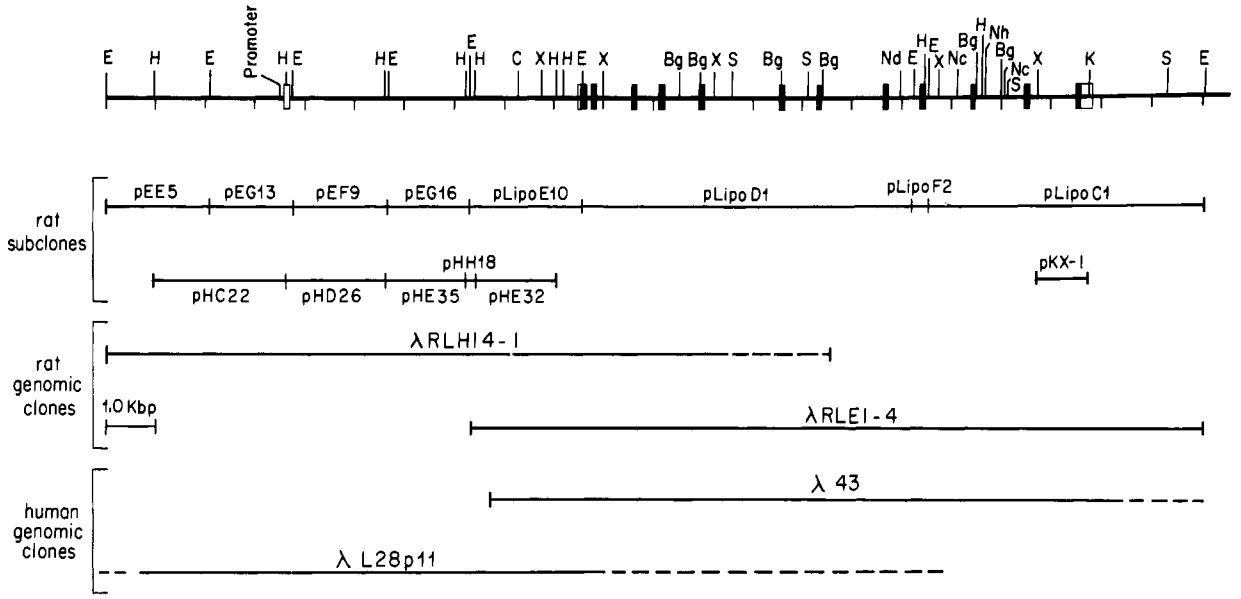

FIGURE 1: Schematic of rat and human lipocortin I genomic clones. The restriction map and the intron sizes refer to the rat gene. The exons containing coding regions are indicated by filled-in boxes, while noncoding exons are represented by open boxes. Sequences of the full-length rat or human genomic clones that have been identified by DNA sequencing are indicated by solid lines. Regions determined by restriction enzyme mapping only are drawn in with dotted lines. The restriction sites are abbreviated as follows: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; Nd, *Nde*I; Nh, *Nhe*I; S, *Sac*I; X, *Xba*I.

λRLH14-1 and λRLE1-4 were performed as described under Materials and Methods.

Two human genomic clones, λ43 and λ28p11, as shown in Figure 1, were isolated from two different genomic libraries. Clone λ43 contains exons 2 through 13, and clone λL28p11 contains the promoter region, exon 1, and additional downstream sequences that have not been characterized. Targeted sequencing was performed to determine all intron–exon boundaries, and the promoter region, as described under Materials and Methods. Only limited sequence information of all human introns was obtained by sequencing one strand of DNA, and for that reason sequence data of the human introns will not be presented here.

Comparison of the Human and Rat Lipocortin I Genes. The rat lipocortin I gene spans 18.5 kb and is divided into 13 exons by introns ranging in length from 113 bp to 5.5 kbp (Figure 2). The locations of the introns in the rat gene were identified by comparing the rat genomic sequence to the human cDNA (Wallner et al., 1986), and their lengths were determined either by DNA sequencing or by extensive restriction enzyme mapping. The locations of the introns in the human gene were determined by targeted sequencing (data not shown) outward from restriction sites within regions corresponding to the centers of the 13 rat exons. The intron–exon boundaries in the human gene were at the same location as in the rat gene.

The exons in both genes can be grouped into four size classes: exons 1, 8, 11 (61, 59, 57 bp); exons 2, 7 (77, 80 bp); exons 4, 6, 9, 10 (95, 91, 94, 96 bp); exons 3, 5, 12 (109, 114, 123 bp). Exon 13 is unique, with a size of 341 base pairs of which 56 base pairs are coding sequences. Exon 1 contains only untranslated sequences; the initiating ATG is located 14 bp into exon 2 in both genes.

Overall the two genes share 87% homology in their coding regions. The transcriptional start sites for both genes are located at the same position. The 5′ flanking sequences show extensive sequence homology, 81% over the first 163 bp upstream of the transcriptional start site (Figure 3). A TATA box and a CCAAT box are located at the same position in both genes, at −30 and −70 bp, respectively. The CCAAT box in the rat promoter diverges from the consensus sequence in that the second A is replaced by a G. The transcriptional start sites for both genes were determined by primer extension of rat or human poly(A) RNA (data not shown) and are located at identical positions (Figure 3) in both promoters.

A 22 bp long sequence (GGAAATAAAAGTGCAAAATAAA) located at position −265 in the human promoter is repeated at position −231 (Figure 3). The motif GGAAA contained in these repeats and found also at position −205 is present in several well-characterized enhancer sequences and in distinct response elements [for references, see Porter et al. (1988)]. A consensus nucleotide sequence for glucocorticoid-receptor binding (TACAATATTTTGTTCT) (Scheidereit et al., 1986) is located in intron 1 at base pair +246 of the human gene. A similar sequence is found in reverse orientation at base pair +183 in the first intron of the rat gene. Glucocorticoid receptor binding studies will have to confirm the functionality of these putative glucocorticoid receptor binding sites.

Relationship between Protein and Gene Structure. The primary structure of lipocortins shows 4-fold internal repeat of 70 amino acids; this domain is repeated eight times in lipocortin VI (p68) (Crompton et al., 1988). In Figure 4 the human and rat lipocortin I amino acid sequences have been divided into the four domains and the N-terminus and have been aligned with the protein sequence of mouse lipocortin II (p36, annexin II). The intron–exon structure of the mouse lipocortin II gene, recently published by Amiguet et al. (1990), is completely conserved between the rat and human lipocortin I genes, with the exception of a slight shift in introns 2, 7, and 12. The conservation of these splice sites is also reflected in the amino acid sequence residues at the intron–exon junctions, which are almost identical in all three genes.

Each domain of the lipocortin proteins contains a highly conserved sequence of 14 amino acids which has been implicated in the binding of calcium (Weber & Johnsson, 1986). Each of these four putative calcium-binding regions is contained within individual distinct exons in the lipocortin I genes (exons 4, 6, 9, and 12 in Figure 4). A second highly conserved sequence has been identified within the domains of other li-

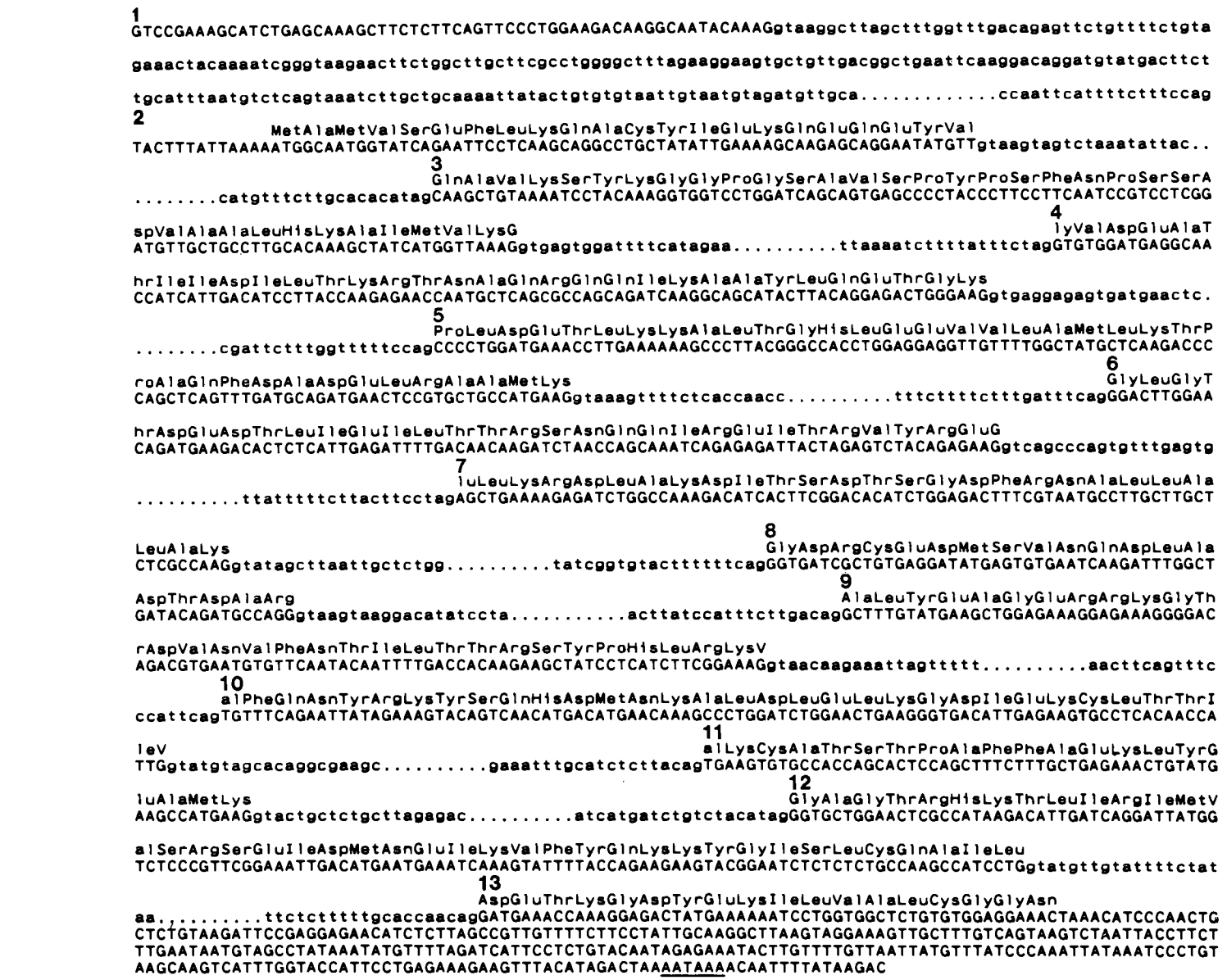

FIGURE 2: Rat lipocortin I gene sequences. Sequences in upper-case letters indicate exon sequences; lower-case letters represent the intron sequences. Only the intron sequences adjacent to the splice junctions are presented. The poly(A) addition signal is underlined. Numbers above the lines denote exons.

pocortins and may be involved in the interaction with phospholipids. Each of these 10 amino acid long repeats is contained within a separate exon (exons 5, 7, 10, 13) located 3′ to the exon coding for the putative calcium-binding region. Thus the four domains which form the core structure are encoded by exons 4 through 13 of the lipocortin I gene.

The 35 amino acid sequence of the lipocortin I amino terminus is completely coded for by exons 2 and 3. This sequence contains a tyrosine residue and a serine residue (at positions 21 and 27, respectively) which are phosphorylated by a variety of protein kinases (Ando et al., 1989) and represents a potential regulatory domain. Lipocortin II has a similar N-terminus with functional properties also regulated by phosphorylation. The N-terminal sequence which is coded for by exons 2 in lipocortin I and II is absent in all other lipocortins characterized so far.

Discussion

To correlate the structural feature of lipocortin I protein with its gene structure, as well as to investigate the mechanisms by which lipocortin gene expression is regulated, we have isolated both the human and the rat lipocortin I genes. The two genes have an identical intron–exon arrangement and highly conserved promoter regions. The gene structure of mouse lipocortin II recently published by Amiguet et al. (1990) and the locations of the splice sites are very similar to those found for human and rat lipocortin I. The protein sequence of rat and human lipocortin I is 89% homologous, and this homology is also conserved between species in the other lipocortin family members (Tamaki et al., 1987; Sakata et al., 1988; Horseman, 1989; Saris et al., 1986; Pepinsky et al., 1988). As discussed below, the structure of the lipocortin I protein and its gene indicates that it has evolved through a duplication of a functional domain.

The lipocortin I protein can be divided into an N-terminal region and a 32-kDa core sequence which consists of four 70 amino acid repeat units (Geisow, 1986b; Pepinsky et al., 1988). A sequence of 14 amino acids is highly conserved within each domain in all lipocortins isolated so far and has been implicated as part of the calcium-binding site. Here we have shown that these putative calcium-binding sequences are located within four individual exons of the rat and human lipocortin I genes.

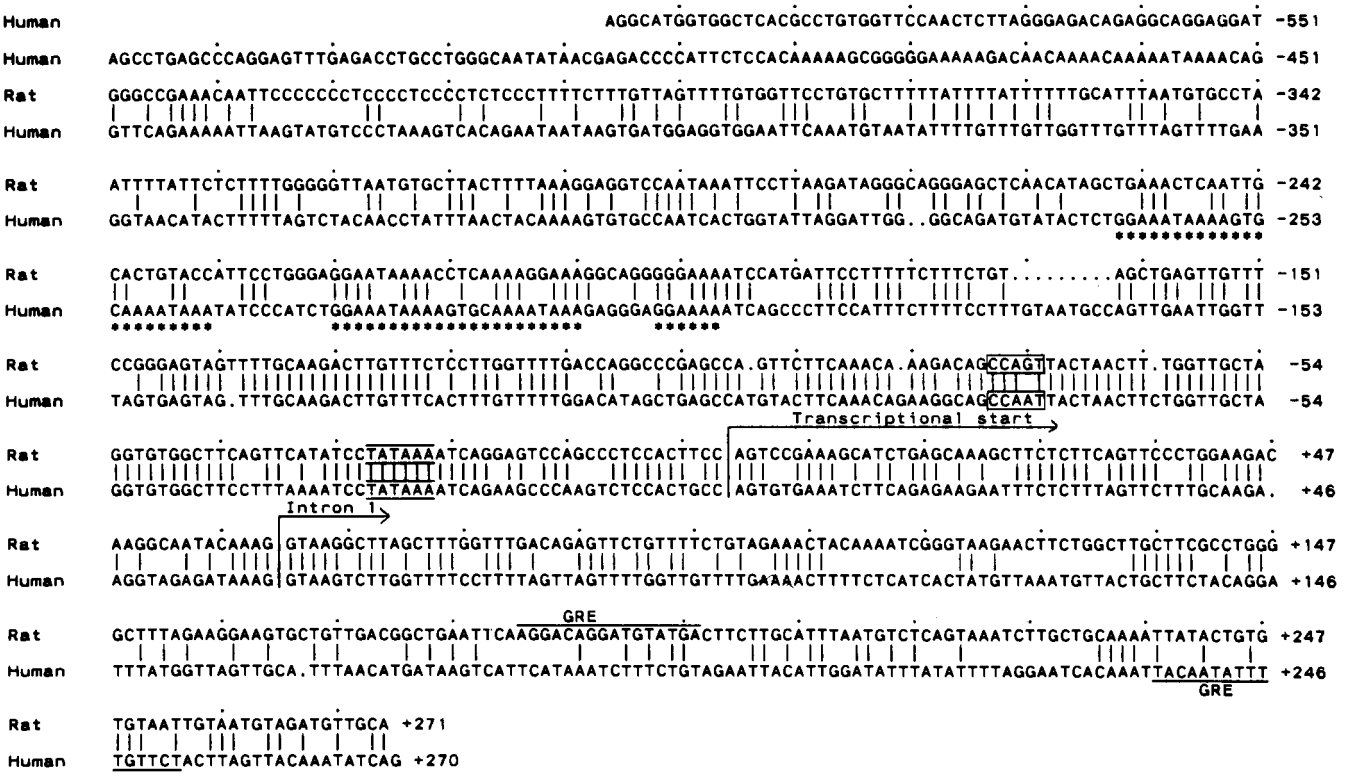

FIGURE 3: Comparison of the rat and human lipocortin I promoter sequences. The TATA and the CAAT sequences are boxed in. The glucocorticoid responsive element GRE sequence in the rat gene is indicated by a solid line over the sequence, and the GRE of the human gene is underlined. The repeated sequences in the promoter regions are marked by asterisks. The transcriptional start in both genes is counted as base pair 1.

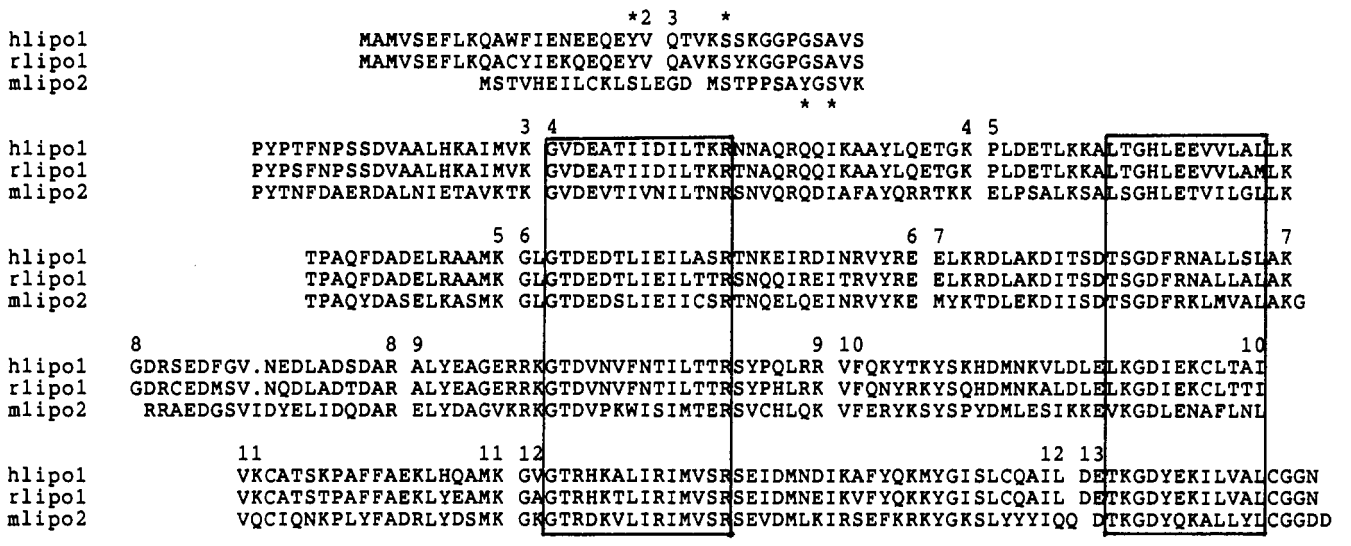

FIGURE 4: Comparison of the human (Wallner et al., 1986) and rat lipocortin I and murine lipocortin II (Amiguet et al., 1990) amino acid sequences. The amino acid sequence of the rat lipocortin I was deduced from the DNA sequence of the assembled rat exons. The tyrosine and serine phosphorylation sites of the N-terminus are indicated by an asterik. The blank space denotes the location of an intron; the numbers above the lines indicate exons. The highly conserved sequences are boxed in.

Each of these exons is followed by an exon containing the second highly conserved region that may be involved in calcium/phospholipid binding (Geisow, 1986b).

The pair of exons containing these highly conserved regions is repeated four times within the lipocortin I gene. This exon–intron structure suggests that the gene evolved through duplication of one functional domain, which consists of two exons. A careful comparison of the protein sequences encoded by the individual exons reveals that exons 3, 4, and 5, which code for the first repeated domain, have a high degree of homology with exons 8, 9, and 10 (coding for the third domain). On the other hand, the second and fourth domains (encoded by exons 6 and 7 and exons 11, 12, and 13, respectively) have striking sequence homology to each other. These observations lead to one possible scenario for how the lipocortin genes evolved. This is depicted in Figure 5. In this model exons 11, 12, and 13, representing the primordial functional domain unit, duplicated successively to form the characteristic 4-fold domain structure of lipocortins I and II. Since exon 5 shows homology to both exons 10 and 11, it appears that exon 5 originated from exons 10 and 11 and subsequently lost the intron. After the duplications, some of the intron–exon boundaries have changed, which accounts for the fact that each domain is not precisely defined. A further

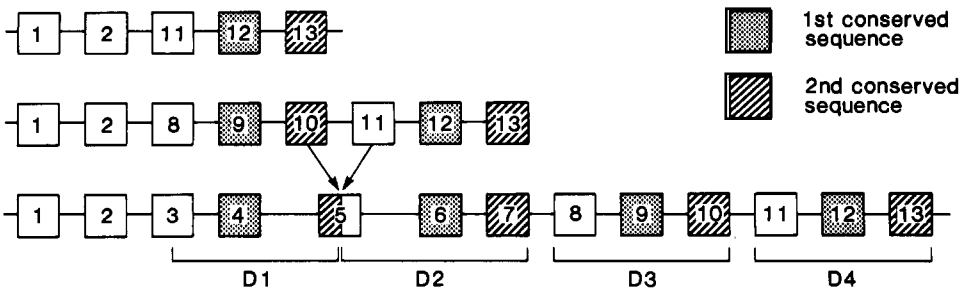

FIGURE 5: Schematic representation of the hypothesized gene duplication of lipocortin I. D stands for repeated domains of lipocortin I.

duplication of the lipocortin I gene must have created a protein with eight repeated domains, p68 (p67 or lipocortin VI). The cDNA of p68 (Crompton et al., 1988; Weber et al., 1987) has recently been isolated, and a comparison of the amino acid sequence to other lipocortins strongly supports the duplication hypothesis (Pepinsky et al., 1988).

The characterization of the mouse lipocortin II gene has recently been reported. Its intron–exon structure is almost identical with that of the human lipocortin I gene (Amiguet et al., 1990).

The amino termini of the lipocortins are distinct from the core structure and show very low sequence homology (Pepinsky et al., 1988). In the lipocortin I gene this sequence is coded for mainly by exon 2. The amino-terminal regions in both lipocortin I and lipocortin II contain sites for phosphorylation, are susceptible to proteolysis, and have been implicated in the regulation of lipocortin activity (Huang et al., 1986: Ando et al., 1989; Glenney & Tak, 1985). The absence of homology in the amino-terminal sequences in other lipocortins suggests that differential regulation of functional activities and target interactions are controlled by these regions. A further argument for independent regulation and functional specificity of these proteins is the differential expression of lipocortins I, II, III, and V in rat tissues and in a variety of established cell lines (Pepinsky et al., 1988). Whether exon 1, which contains the 5′ untranslated region, and exon 2, which codes for the nonconserved N-terminal sequence of lipocortin I, were lost in other members of the lipocortin family or acquired in lipocortins I and II after divergence of these genes will become clear through comparison of all lipocortin gene structures.

Induction of lipocortin I and lipocortin II transcription by dexamethasone has been demonstrated in several tissues, in macrophages in vivo, and in primary cell cultures (Wallner et al., 1986; Wong et al., 1991; B. Wallner, unpublished observations). Regulation of gene expression by steroids involves the interaction of the glucocorticoid receptor–steroid complex with specific transcription factors and glucocorticoid regulatory elements (GRE) located in the vicinity of the promoter of the target gene. A comparison of these sequences in a large number of steroid-induced genes has revealed a highly conserved hexanucleotide sequence (Scheidereit et al., 1986). One perfect match with this consensus sequence is located in the first intron of both the rat and human lipocortin I gene. This is similar to the location of the functional GRE in the human growth hormone gene (Slater et al., 1985). Whether GREs in the lipocortin I genes are functional will have to be confirmed by biological studies.

A comparison of the sequences in the vicinity of the rat and human lipocortin I promoters revealed no striking sequence homologies to consensus sequences of transcriptional binding factors. However, an exact repeat of 22 base pairs in the human promoter contains the motif GGAAA, which is found in well-characterized response elements and enhancer sequences recognized by transcription factors which are regulated by agents such as interferons, mitogens, and serum factors (Porter et al., 1988). Lipocortin I transcription is increased by TPA, γ-IFN, and serum (B. Wallner, unpublished observation). It will therefore be of interest to determine whether these sequences are directly involved in the regulation of lipocortin I gene expression.

Our current knowledge indicates that both lipocortin I and lipocortin II genes have evolved from a common ancestral gene, which itself arose through two rounds of duplication of a functional domain. When the intron–exon structure of other members are known, it will be possible to determine whether they have evolved from one ancestral gene or arose through duplications of one functional unit. The high degree of amino acid sequence homology at several splice junctions in all lipocortins isolated so far strongly indicates conservation of the exon–intron structure.

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