Calretinin: A Gene for a Novel Calcium-binding Protein Expressed Principally in Neurons

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Abstract. A novel gene of the calmodulin superfamily, encoding a 29-kD neuronal protein here named "calretinin," has been isolated as a cDNA clone from chick retina. The encoded sequence includes four putative calcium-binding sites and a fusion protein binds calcium. The most similar protein known is the 28-kD intestinal calcium-binding protein, calbindin (58% homology). Both genes date from before the divergence of chicks from mammals. The distribution of calretinin and calbindin mRNAs in chick tissues has been mapped using RNA gel blots and in situ hybridization. RNAs from both genes are abundant in the retina and in many areas of the brain, but calretinin RNA is absent from intestine and other nonneural tissues. Calretinin and calbindin are expressed in different sets of neurons throughout the brain. Calretinin RNA is particularly abundant in auditory neurons with precisely timed discharges.

THE superfamily of calcium-binding proteins, whose prototype is calmodulin, contains at least a dozen members which perform a wide variety of calcium-dependent functions in different tissues. Calmodulin itself regulates several important enzymes in most cell types (34). Troponin C and the myosin light chains regulate muscle contraction (1). Parvalbumin, another muscle protein, appears to be involved in terminating fast contractions (26). Another important member of the superfamily is the 28-kD vitamin D–dependent intestinal calcium-binding protein (70), also known as cholecalcin 28k, spot 35, or visinin (47a), and now re-named calbindin D_{28k}—hereafter "calbindin." Calbindin appears to be involved in intestinal calcium transport, but is also present in kidney, bone, and several other tissues (30, 46, 65). The calbindin gene has been cloned by several groups (21, 27, 28, 71, 72).

Many neurons in the brains of mammals and of birds exhibit immunoreactivity for parvalbumin (7, 9, 13, 15, 17, 18) or for calbindin (5, 20, 22, 29, 30, 33, 57). Each is present only in some neurons, commonly throughout the whole neuron including the dendritic tree and axonal terminals. They are both very abundant in the Purkinje cells of the cerebellum, and calbindin constitutes ~15% of the soluble protein of these cells (5, 6, 30, 40, 47, 64). But elsewhere in the brain, calbindin and parvalbumin immunoreactivities are mostly in separate neurons (9, 11, 24). Both are also present in rat retina (17, 50), and calbindin immunoreactivity is present in chick retina (25, 53, 58).

The function of these proteins in neurons is unknown. An initial suggestion that parvalbumin was colocalized with GABA (15) has not been generally confirmed by more extensive surveys (13, 18). Parvalbumin-containing neurons do generally have rapid firing rates (9, 14). The numerous calbindin-immunoreactive cells are not known to have any common features.

I now report the isolation from chick retina of an mRNA encoding a new member of the superfamily, which is expressed in the retina and in brain neurons. Because of its homology with calcium-binding proteins and its tissue of origin, I refer to the encoded protein as calretinin. The apparent calbindin immunoreactivity in some neurons is attributable to calretinin. Calbindin and calretinin are located in distinct sets of neurons.

Materials and Methods

Cloning cDNA

All RNA samples were isolated by the guanidinium thiocyanate method (42) and were poly(A)-selected on oligo(dT)-cellulose unless otherwise stated. Poly(A)$^+$ RNA from retinas of chicks 1–3 d old was used for cDNA cloning. The cDNA first strand was primed with a dT-tailed Eco RI linker, 5′-CCCGAATTCGGG$(T)_n$-3′. After first-strand synthesis, the reaction was boiled for 4 min to destroy the RNA, and hairpin-primed second-strand synthesis and nuclease S1 cleavage were performed as in reference 42. The cDNA was blunt-ended with Klenow enzyme, digested with Eco RI, size selected on a Sepharose-CL4B column, and ligated to M13-mp9 DNA which had been cut with Eco RI plus Hinc II and phosphatased. This procedure should yield inserts with a blunt 5′ junction and an Eco RI 3′ junction. The 3′ junction should be adjacent to the universal primer site, having been formed either from the Eco RI linker/primer or, as probably happened in clone RU37, from an internal Eco RI site in the cDNA.

The ligation mixture was transfected into *Escherichia coli* strain CMK603. To identify retina-specific clones, plaques were screened by hybridization with labeled cDNA to chick retina RNA or to wingbud RNA of 15-d-old chick embryos, prepared with α[^{32}P]dATP to high specific activity. Clone RU37 was one clone positive with retina cDNA only.

Clone Analysis

DNA sequencing was done using the dideoxy technique (56) with α[^{35}S]-dATP and gradient gels (8). All of RU37 was sequenced on both strands.

The chick calbindin cDNA clone pWH11 (71) was given by P. Wilson and E. Lawson, along with two subclones of it in M13 phage, SS58 (coding sequence) and SS10 (3′ untranslated sequence), both oriented such that a la-

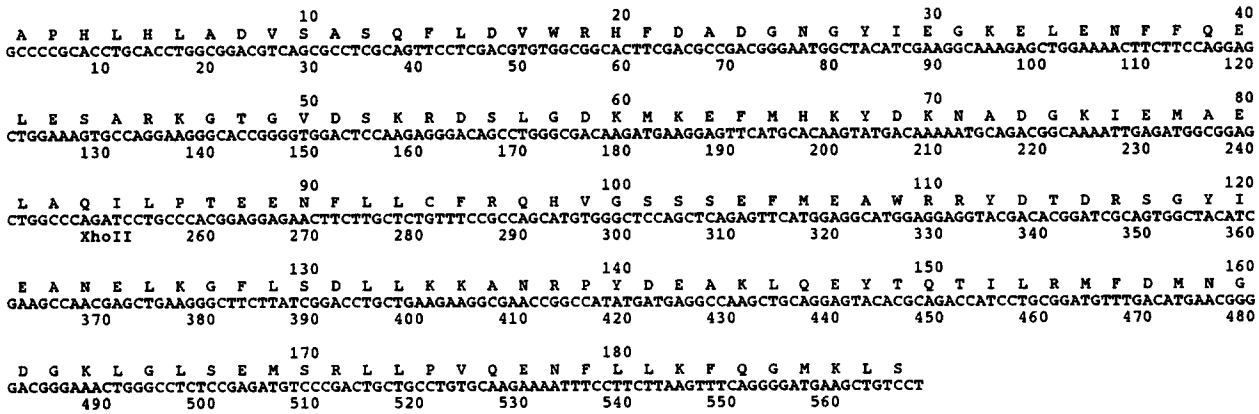


Figure 1. Sequence of cDNA insert in clone RU37.[2]

beled complementary strand would hybridize to the calbindin mRNA. It is clear from the results that the calretinin and calbindin genes do not cross-hybridize under the conditions used.

DNA gel blots used the method of Southern (59). RNA gel blots used formaldehyde gels (42), blotted onto nitrocellulose immediately after electrophoresis. The probes were labeled with ^{32}P either by nick-translation or by single-strand synthesis (2). RNA gel blots were hybridized (66) at 42°C, and were washed in 0.1× standard saline citrate (SSC)-0.15 NaCl, 15 mM sodium citrate, pH, 7.0, 0.1% SDS, at 50°C. DNA gel blots were hybridized similarly, but at 37°C, and were washed in 2× SSC, 0.1% SDS at 50°C.

In situ Hybridization (ISH)[1]

ISH was performed with single-stranded DNA probes, labeled with ^{35}S plus a trace of ^{32}P, as previously described (52). After hybridization, sections were washed in 2× SSC at room temperature, and some also in 0.1× SSC at 50°C as for the gel blots; there was no significant difference in the results. Calbindin probes SS58 and SS10 gave similar results.

Most of the results shown here were obtained with the brain from a 1.5-d-old chick which had been perfused with 4% paraformaldehyde. The major positive areas were also recorded in other, fresh-frozen chick brains.

Expression of Fusion Protein

Two fusion proteins were made in the pUR289 vector (55). One contained the whole RU37 insert, excised with polylinker as a Hind III–Eco RI fragment and inserted into the Hind III site. The other contained the 3′ half, from the Xho II site to the 3′ end (Fig. 1), inserted into the Bam HI site. Bacteria containing these plasmids, from 2.5 ml of overnight culture, were resuspended in 0.2 ml of 62 mM Tris, pH 6.8, 0.7 M 2-mercaptoethanol, 10% glycerol, 2.3% SDS, 0.001% bromophenol blue, and were lysed by sonication, spun to remove debris, and boiled for 10 min. Samples of 4 μl were run on 9.5% SDS-PAGE. Electroblotting and ^{45}Ca-binding were done as in Maruyama et al. (43).

In Vitro Translation

In vitro translation, immunoprecipitation, and SDS-PAGE were done as in reference 19. For immunoprecipitation, 0.5 μg of poly(A)$^+$ RNA was translated in reticulocyte lysate. One third of the reaction product was then incubated with antiserum against calbindin (provided by Dr. D. E. M. Lawson) and one third with normal rabbit serum. Immunoprecipitates were recovered using Protein A–Sepharose CL4B (Pharmacia Fine Chemicals, Uppsala, Sweden), and were subjected to electrophoresis alongside portions of the unfractionated translation products.

For hybrid selection of mRNA, several micrograms of M13 phage DNA carrying an insert complementary to RU37 were boiled, made up to 2× SSC, spotted onto small squares of nitrocellulose, and baked. Hybrid selection was done with 10 μg of poly(A)$^+$ RNA, as in reference 51. Eluted RNA was translated in reticulocyte lysate and the products were subjected to electrophoresis as above.

Results

The Calretinin Gene

A small cDNA library was made from chick retina RNA in an M13 phage vector as described in Materials and Methods, and was screened for retina clones by plaque hybridization with ^{32}P-labeled cDNA from retina and from embryo wing-bud. One of the clones identified as retina-specific by this criterion was RU37. It was sequenced, and found to contain a 568-nucleotide insert with a continuous open reading frame (Fig. 1). A computer library search identified the encoded sequence as a member of the calmodulin superfamily

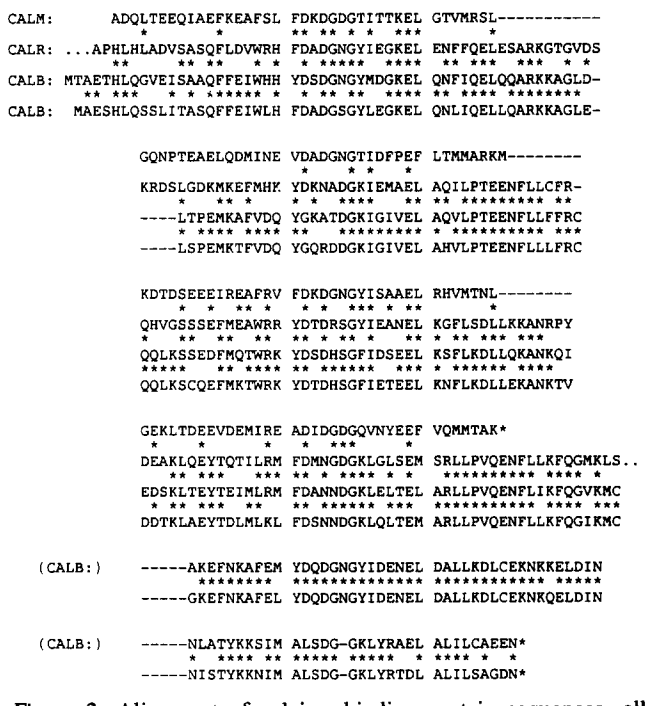


Figure 2. Alignment of calcium-binding protein sequences, all translated from cDNA. *CALM*, calmodulin (34). *CALR*, calretinin (Fig. 1). *CALB*, calbindin. The upper row is chick calbindin (71); the lower row is rat calbindin (72).

1. *Abbreviation used in this paper*: ISH, in situ hybridization.

2. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00625.

of calcium-binding proteins. The closest homology is with calbindin (Fig. 2). When the two sequences are aligned, the RU37 insert begins a few codons after the calbindin initiation codon, and ends after the fourth of the six sites which match the canonical calcium-binding site sequence. From the method of clone construction, this 3′ truncation may be due to an internal Eco RI site.

The sequence implied that the encoded protein, named calretinin, should bind calcium. To test this, two fusion proteins were made in the pUR289 vector. One contained the whole RU37 insert, and the other contained domains III and IV. Lysates of bacteria expressing these β-galactosidase-calretinin fusion proteins were run on SDS–PAGE and assayed for binding of ^{45}Ca. Both fusion proteins bound ^{45}Ca whereas β-galactosidase itself did not (Fig. 3).

On chick genomic DNA blots, ^{32}P-labeled RU37 insert lights up two bands with Bam HI and one band with Hind III. A clone of the chromosomal gene corresponding to RU37 (Rogers, J., and M. Goedert, unpublished data) contains a site for Bam HI within an intron interrupting the cDNA sequence. It is therefore likely that RU37 hybridizes to only a single gene in the chick. It also hybridizes faintly to a pair of bands in rat and guinea pig DNA at low stringency.

Similar genomic DNA blots were hybridized with a probe for the calbindin gene, pWH11 (see Materials and Methods). This lights up one or more bands in chick and (faintly) one band in rat, which are different from those seen with RU37. Thus the probes for calretinin and calbindin do not cross-hybridize even under these low-stringency conditions, and each corresponds to a separate gene in chicks and probably in mammals.

Calretinin and Calbindin mRNAs

Blots of RNA from various chick tissues were hybridized with probes for calretinin and calbindin (Fig. 4). In retina and in the brain, the calretinin probe detected major RNAs of 1.4 and 1.2 kb, and the calbindin probes detected major RNAs of ~3.2 and 2.3 kb. The same calbindin RNAs were present in intestine and kidney, and are presumably the same as those of 3.1 and 2.0 kb seen by others (27, 32). Calretinin RNAs, on the other hand, were absent from intestine and kidney, and also from the other nonneural tissues listed in the Fig. 4 legend. The only nonneural tissue in which traces of calretinin RNA were found was bone. Several preparations of RNA from bone (femur of day-old chick including marrow and traces of muscle), which appeared to be intact on gels, all displayed a low molecular weight smear of hybridization with the calretinin probe. This suggests that calretinin may be expressed in some cell type in bone which is exceptionally prone to autolysis during extraction, perhaps osteoclasts.

Within the brain, the calbindin RNAs were found in all regions and were most abundant in the cerebellum, which is known to be a major source of calbindin. The calretinin RNAs were also found in all regions but were comparatively rare in cerebellum and cerebral hemispheres; they were most abundant in the retina.

Cellular Distribution of Calretinin and Calbindin mRNAs

To identify the cells expressing each gene, ISH was performed on chick brain sections using ^{35}S-labeled single-stranded DNA probes specific for each gene. The results showed that calretinin and calbindin are generally, if not entirely, in different cells (Table I and Fig. 5). All the positive cells appear to be neurons.

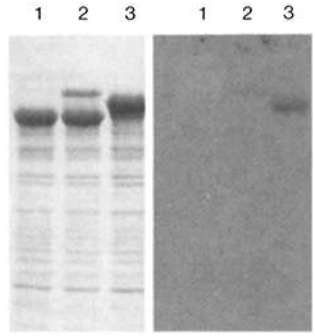


Figure 3. β-Galactosidase-calretinin fusion proteins bind calcium. Lysates of bacteria containing plasmids were run on SDS–PAGE; the left panel was stained with Coomassie Blue, the right panel blotted and incubated with ^{45}Ca (42). (Lane *1*) Vector pUR289 (β-galactosidase from the plasmid is the most densely stained band); (lane *2*) fusion pUR37.1, containing the whole RU37 insert (the fusion protein is partly degraded to β-galactosidase); (lane *3*) pURXR.3, containing RU37 calcium-binding sites III and IV (from the Xho II site to the 3′ end in Fig. 1). Note that the fusion proteins bind ^{45}Ca but β-galactosidase does not.

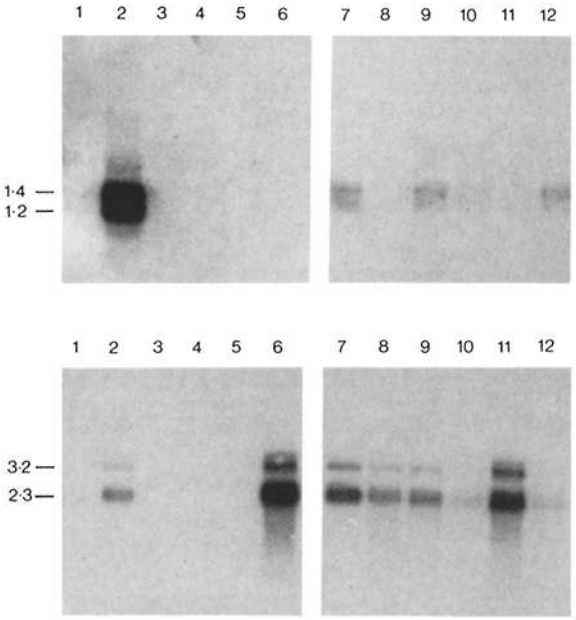


Figure 4. RNA gel blots hybridized with calretinin and calbindin probes. (*Upper*) Calretinin: single-stranded probe from clone RU37. Left panel exposed for 4 d at −70°C with intensification screen; right panel exposed overnight at room temperature. (*Lower*) Calbindin: nick-translated probe pWH11. Both panels exposed overnight at −70° with intensification screen. Lane *1* contains 6 μg of poly(A)$^-$ retina RNA. The other lanes contain poly(A)$^+$ RNA, 5 μg, from tissues of chicks 1–2-d old unless stated otherwise, as follows: (lane *2*) retina, 3 μg; (lane *3*) liver, 3 μg; (lane *4*) wingbud of 15-d-old embryo, 3 μg; (lane *5*) muscle, 5 μg; (lane *6*) small intestine, 5 μg; (lane *7*) retina, 3.5 μg; (lane *8*) telencephalon; (lane *9*) diencephalon plus mesencephalon except optic lobes; (lane *10*) optic lobes; (lane *11*) cerebellum; (lane *12*) pons plus medulla. RNA gel blots were also performed with the following total RNAs, 20 μg per lane, and were negative for calretinin, under conditions where it was readily detected in retina and spinal cord RNA: kidney, duodenum, gizzard, spleen, heart.

Table I. Positive Regions for CaBP RNA and Immunoreactivity in Chick Brain

	Abbreviation		Calretinin ISH (RU37)		Calbindin ISH (SS58,SS10)		Calbindin immunoreactivity Roth et al.		Jande et al.
Telencephalon									
Hyperstriatum	HV,HD,HA		−		++ s		+		+++ s
Neostriatum	N		−		++ s		+		+++ s
Archistriatum	Av,Ad		−		+ s		+		
Nuc. septalis med.	SM		−		+ s		+		
Palaeostriatum:									
augmentatum	PA	(periph.)	+ s		(+ ss)		+		+
primitivum	PP		+		(+ ss)				
Diencephalon									
Nuc. rotundus & triangularis	Rt, T		−		++		+		+
Nuc. spiriformis med.	SpM		++		−				+
Nuc. spiriformis lat.	SpL		−		++				?++
Nuc. lat. ant. thalami	LA		+		−		+		
Nuc. ventrolateralis thalami	VLT		(± s)		+ s				+++ s
Nuc. geniculatus lat. (dorsalis)	GLd		+		−		+		
Nuc. posteroventralis thalami	PV		+ s		nd		−		
Nuc. subrotundus	SRt		++		−				
Habenula*		(HM)	+	(HL)	++	(HL)	+	(HM)	+++
Dorsal thalamus‡									
Nuc. dorsolateralis	DL	(SMe,SPC)	++	(AP,DLP)	++	(DLP)	+	(AP,SHL)	++ s
		(DLL)	++						
Nuc. dorsomedialis	DM		−		+		+		
Hypothalamus:§									
Nuc. periventricularis mag.	PVM		−		++		+		+++
Nuc. med. hypothalami post.	PMH		−		++		+		++ s
Dorsolateral part		(SCE,AL)	(+ ss)		(+ ss)	(AL)	+	(AHP)	++ s
Ventrolateral part		(SCI)	++ s	(SCI,PLH)	++ s	(PLH)	+	(SCI)	++ s
		(LHy-lat.)	++ s	(LHy,AVT)	++ s			(ML,AVT)	++ s
Nuc. ant. med. hypothalami	AM		−		+ s		+		
Nuc. ectomamillaris	EM		+		−		+		+
Mesencephalon									
Locus coeruleus	LoC	(ventral)	(+ sg)		−		+		
Nuc. semilunaris	SLu		++		−				
Nuc. ventralis lemnisci lat.	VLV		(±)		−				+++
Reticular formation‖	Ret	(SCv,Ret)	++ s	(in SCv,FRL)	(+ sg)	(Ret)	+	(SCv)	+ s
Optic lobes:									
Tectum	TeO	(11–13)	(±) s	(5–6)	+	(5–6)	+		++ s
Nuc. intercollicularis	ICo		+ s		+s	[MLd-lat.]	(+ sg)		+ s
Nuc. isthmi (mag.)	Imc		++		−		+		++
Nuc. lentiformis mesencephali (mag.)	LMmc		++ s		−				
Nuc. principalis precommissuralis	PPC		+ s		−				
Cerebellum									
Purkinje cells			−		+++		+		+++
Metencephalon									
Nuc. angularis	An		++		−		−		
Nuc. sens. princ. nervi trigemini	Pr*V*		++		−				+
Nuc. vestibularis superior	VS		++		−		−		
Nuc. vestibularis lat.	VeL		++ s		−		−		
Nuc. vestibularis med.	VeM		(++ ss)		(+ ss)		(+ ss)		
Nuc. vestibularis descendens	VeD		+ s		(+ ss)		(+ ss)		+++ s
Nuc. laminaris	La		++		−		−		++
Nuc. mag.	MC		++		−		−		++
Nuc. nervi facialis	n*VII*		+		−		−		
Nuc. paragigantocellularis lat.	PGL	(OI-lat.)	++						
Nuc. olivaris inferior	OI		−		++		+		++
Nuc. solitarius	S		+ s		++		+		+++
Nuc. motorius dorsalis nervi vagi	n*X*		+		−		−		
Dorsolateral regions¶		(TTD,Ta)	+ s	(in TTD)	(++ sg)	(TTD)	+	(Ta,ST)	++
				(CE)	(+ ss)	(CE)	+		
Ventral regions‖	Ret	(all)	++ s	(FRL)	(+ ss)	(Ret)	(+ ss)	(FRL)	+ s
*Spinal cord***									
Dorsal regions		(med.)	(+ sg)	(TTD)	+ s	(TTD,DH)	+	(SG)	+
Lateral regions			(+ sg)		−			(ST)	++
Vestibular ganglion‡‡			+		+				*XX*
Retina§§									
Photoreceptor layer	PRL		+		+			(cones)	*XX*
Inner nuclear layer	INL (outer edge)		+		(±)			(horizontals)	*XX*
Inner nuclear layer	INL (inner edge)		+		? (± ss)			(amacrines)	*XX*
Ganglion cell layer	GCL								

Calbindin-positive cells predominate in the cerebral hemispheres, optic tectum, and cerebellum. In the cerebral hemispheres (Fig. 6, 7 *e*), calbindin is present in medium-sized cells (~13 μm) scattered all through the hemispheres, except in the palaeostriatum where they are very sparse. In contrast, calretinin is seen only in the palaeostriatum—in many cells in PP and in a narrow band of cells around the periphery of PA. In the tectum, calbindin is present in many cells of layer 5 and some of layer 6 (Fig. 7 *f*); calretinin is present only weakly, if at all, in the deeper layers. In the cerebellum, as expected, calbindin is very abundant in the Purkinje cells (Fig. 7 *h*), while calretinin is totally absent. (The slight signal on the RNA gel blot with calretinin is attributable to the calretinin–positive cells at the root of the cerebellum in the nucleus angularis and brachium conjunctivum.)

Of the major nuclei of the brain, some are strongly positive with calretinin and some with calbindin, while others express neither (Table I). The major calretinin-positive nuclei are isthmi magnocellularis (Fig. 7 *a*), spiriformis medialis, subrotundus, dorsolateralis anterior thalami, semilunaris, principalis nervi trigemini, laminaris, and magnocellularis. The major calbindin-positive nuclei are rotundus, spiriformis lateralis, habenula lateralis, and the inferior olive. In most of these, the signal is on all or (in rotundus, Fig. 7 *g*) on most of the large neurons that make up the nucleus.

The only nuclei which show similar patterns of expression with both genes are solitarius (Fig. 7 *d*) and intercollicularis, as well as the vestibular ganglion which was included on some sections. In nuclei solitarius and intercollicularis, and the vestibular ganglion, neither probe lights up all the cells, and these nuclei are each known to contain neurons of several types, so it is entirely possible that calretinin and calbindin are expressed in different cells.

In the more complex regions of the brainstem, there are positive cells with both probes, but their distribution is clearly different. In the hypothalamus, calretinin-positive cells predominate in the ventrolateral areas, while calbindin-positive cells are present throughout the hypothalamus, and especially in the medial nuclei. In the reticular formation of mid- and hind-brain, there is a similar contrast in the distribution of positive cells, which are moderately large neurons (14–20 μm) (although the largest neurons are negative). Calretinin-positive cells are widespread throughout the reticular formation of the midbrain and pons, and virtually absent from the lower medulla. Calbindin-positive cells are rare in the midbrain and pons, but abundant in the dorsal half of the lower medulla. Although the possibility of co-expression in a few cells cannot be excluded, these data indicate that calretinin and calbindin are generally in different populations of neurons.

In the chick retina, cells are too closely packed to be resolved by ISH, but calretinin and calbindin RNAs do show different distributions (Fig. 8). Calretinin signal is seen in all three nuclear layers. Calbindin signal is strong only on the photoreceptor layer; scattered groups of grains may indicate hybridization to scattered cells in the inner nuclear layer and ganglion cell layer.

In Table I, the ISH results are compared with published distributions of calbindin immunoreactivity. The immunoreactivity reported by Roth et al. (54) generally coincides with the calbindin RNA, but the presence of immunoreactivity in nucleus isthmi magnocellularis and a few other sites, which are positive for calretinin RNA but negative for calbindin RNA, suggests that the antiserum cross-reacted weakly with calretinin. The immunoreactivity reported by Jande et al. (29) coincides with the calbindin RNA and the calretinin RNA, indicating that their antiserum must have efficiently detected both proteins under their conditions. The probable basis of this cross-reaction is discussed below.

Identification of Calretinin Protein

Calbindin antiserum immunoprecipitates a 29-kD protein, as well as 28-kD calbindin itself, from translation products of brain mRNA (49). As shown in Fig. 9 *a* (lanes *3–6*), the 29-kD band is more abundant than calbindin in the translation products of chick retina and midbrain mRNA, but only calbindin is produced from cerebellum mRNA, consistent with the distributions of calretinin and calbindin RNAs reported above. The following experiment was therefore done to see whether the 29-kD band was calretinin.

Messenger RNA from retina and from midbrain was hybrid selected with an M13 subclone complementary to the RU37 sequence, then translated in reticulocyte lysate. The hybrid-selected mRNAs produced a 29-kD polypeptide (Fig. 9 *b*, lane 7). This is identical in size to the upper band immunoprecipitated by calbindin antiserum. These results strongly indicate that this 29-kD band is calretinin.

The first two data columns give the results from ISH. The third and fourth columns list all the areas showing calbindin immunoreactivity according to Roth et al. (54) and Jande et al. (29), except for a few midbrain nuclei which were not examined by ISH.

Abbreviations are from references 31 and 73. Other abbreviations: *s*, scattered cells; *ss*, sparsely scattered cells; *sg*, small group of cells; *ant.*, anterior; *post.*, posterior; *med.*, medialis; *lat.*, lateralis; *mag.*, magnocellularis.

In some regions, named areas are contiguous and poorly distinguished in the atlases, so different names may be used by different authors. Details of these regions are as follows.

* Habenula. Calretinin and calbindin RNAs are in different nuclei. *HM*, habenula med.; *HL*, habenula lat.

‡ Dorsal thalamus. Calretinin RNA is in the most dorsal areas; calbindin in nuclei just ventral. *AP*, area pretectalis; *DLP*, DL post.; *DLL*, DL ant. (lat.); *SHL*, nuc. subhabenularis lat.; *SMe*, stria medullaris; *SPC*, nuc. superficialis parvocellularis.

§ Hypothalamus. Calbindin RNA is in cells which are abundantly scattered throughout most of the hypothalamus; calretinin RNA is in similar cells which are more restricted in distribution, being mainly in the lateral part of LHy. *AHP*, area hypothalami posterior; *AL*, ansa lenticularis; *AVT*, area ventralis (Tsai); *LHy*, nuc. lat. hypothalami; *ML*, nuc. mamillaris lat.; *PLH*, nuc. lat. hypothalami post.; *SCE*, external cellular layer; *SCI*, internal cellular layer.

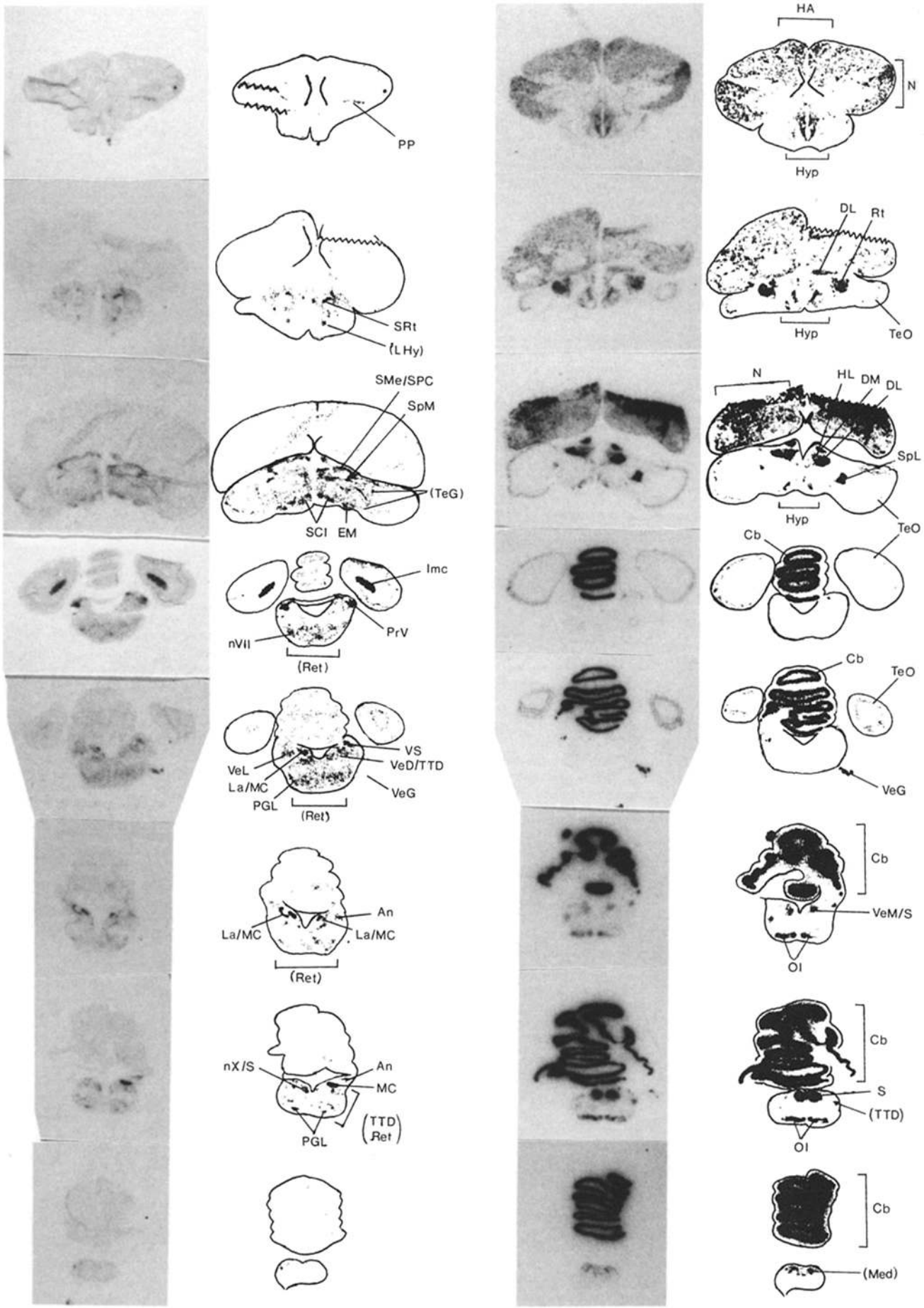
‖ Reticular formation of mid- and hind-brain. Calretinin RNA is in cells that are abundantly scattered through much of the region; calbindin RNA is absent, except for a few cells in lateral regions. *FRL*, lateral reticular formation; *SCv*, nuc. subcoeruleus ventralis.

¶ Dorsolateral part of pons and medulla. Calretinin-positive cells are widespread; calbindin-positive cells are very few. There is a small clump of giant neurons intensely positive for calbindin within TTD. *TTD*, nuc. and tractus descendens nervi trigemini; *Ta*, nuc. tangentialis; *ST*, nuc. subtrigeminalis; *CE*, nuc. cuneatus externus.

** Spinal cord at approximately C1 level. Calbindin-positive cells are widespread, all across the dorsal half, while calretinin-positive cells are restricted to two sparse clusters just ventral to the calbindin-positive cells. The ventral half is negative. *SG*, substantia gelatinosa Rolandi; *DH*, dorsal horn.

‡‡ *XX* Vestibular ganglion immunoreactivity was reported in the cat (57).

§§ *XX* Retina immunoreactivity was reported in the chick by Schreiner et al. (58) (who saw it in all photoreceptors) and by Roman et al. (53) (who saw it in only one class of photoreceptors, the double yellow cones).



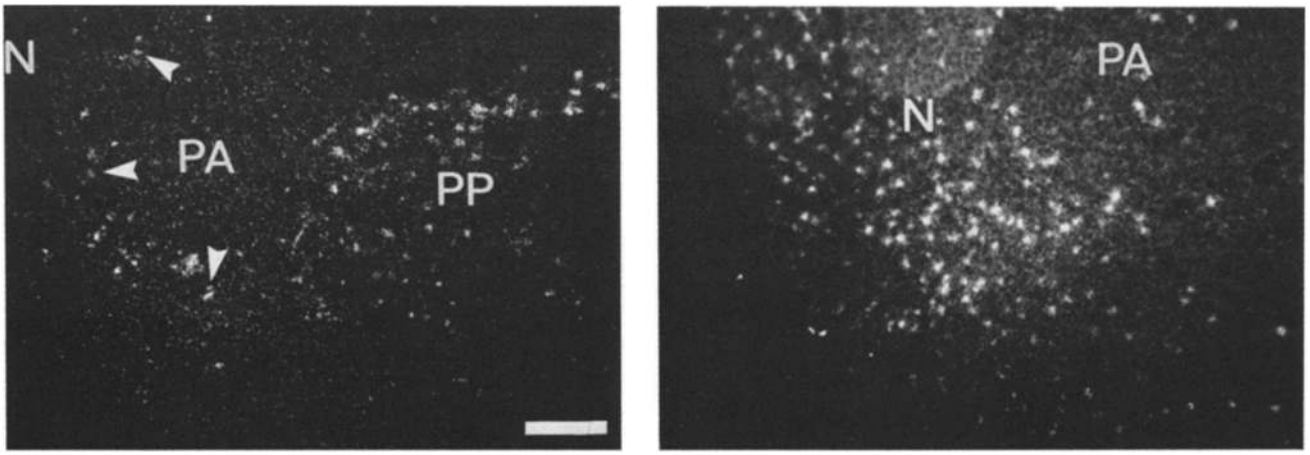


Figure 6. Sections of chick forebrain hybridized with probes for calretinin (*left*) and calbindin (*right*), viewed in dark field. The lateral surface of the brain crosses the left edge of each field and the ventral surface is at the bottom edge. *N*, neostriatum; *PA*, palaeostriatum augmentatum; *PP*, palaeostriatum primitivum. Bar, 200 μm.

Discussion

Homology and Topology of Calretinin and Calbindin

Of all the known members of the calmodulin superfamily, 28-kD calbindin is the most homologous to calretinin and is the only one which aligns with it near continuously, the average homology being 58% (Fig. 2). This is less than the 76% homology of calbindin from chick to that from cow (62) or rat (72), and an analysis of informative residues (not shown) confirms that calretinin and calbindin diverged before the avian and mammalian calbindins. This is consistent with the genomic Southern blots which show that probes for the calretinin and calbindin genes of chick (which do not cross-hybridize) light up different bands in mammals, suggesting that the two genes had been separately conserved in chickens and mammals.

The characteristic calcium-binding site sequence of the calmodulin superfamily folds into a secondary structure called the "EF hand" (36, 37). These calcium-binding sites generally come in pairs, and the pairs form domains in the tertiary structures (4, 38). The predicted partial calretinin sequence contains four putative calcium-binding sites. A computer secondary structure prediction (23) (analysis not shown) indicates that each of them should form a turn or coil between two α-helices, as in calmodulin, except for site II. The algorithm predicts continuous α-helix across site II, suggesting that this site may be aberrant, like site II of calbindin.

Within calretinin and calbindin, sites I and II are most homologous to sites III and IV, respectively. The duplication of this pair of sites apparently occurred in the calbindin/calretinin ancestor independently of the similar duplication in calmodulin (Fig. 10), as all four calmodulin sites are more homologous to calbindin sites I and III than to sites II and IV (Fig. 2; and diagonal matrix plots [not shown]).

The sequences between the calcium-binding sites are longer in calretinin than in most members of the calmodulin superfamily, but are colinear with those of calbindin, except for the stretch between sites I and II. (The sequence of most of that divergent stretch in calretinin, together with the whole of RU37 nucleotides 151–512, has been confirmed by sequencing a genomic clone [Rogers, J., and M. Goedert, unpublished results].)

The most interesting parts of the sequence are those after sites II and IV, where homologous stretches of 14–15 amino acids are virtually identical between calretinin and calbindin, and are also highly conserved in calbindin itself (Fig. 2). Each of these stretches begins in the alpha-helix after the calcium-binding site and extends into a predicted loop (as the secondary structure program predicts a loop and turn, not a continuous helix, between sites II and III). Each conserved stretch is followed by a cysteine (except that the RU37 sequence does not extend that far after the second stretch), but these cysteines are not in exactly corresponding positions, and therefore were probably introduced independently into each of these loops in evolution. By comparison with the structures of other members of the superfamily, it is expected that these conserved stretches will be accessible at the surface of the proteins, where they might interact with some other protein.

These conserved stretches may also be the basis of the serological cross-reactivity between calretinin and calbindin which was inferred from the observed distributions. Because both proteins appear to be conserved in mammals, surveys of calbindin immunoreactivity in mammals (6, 16, 20, 22, 29, 33, 50, 57) may also have detected calretinin as well as calbindin.

The primary translation product of calretinin mRNA is 29 kD, and this is almost certainly the same molecule that is immunoprecipitated by calbindin antisera (Fig. 9). This protein

Figure 5. ISH with probes for calretinin (*left*, probe RU37) and calbindin (*right*, probe SS58), autoradiographed on X-ray film. Abbreviations are as in Table I, plus: *Hyp*, hypothalamus; *VeG*, vestibular ganglion; *Med*, dorsal medulla. Zigzags indicate spurious lines due to tissue folds. The positions of the sections, in millimeters from the rostral pole of the fixed brain, were as follows. Calretinin: 2.3, 3.5, 4.8, 6.7, 7.4, 7.9, 8.3, 9.6; calbindin: 2.9, 3.9, 4.8, 6.8, 7.4, 8.1, 8.6, 9.7.

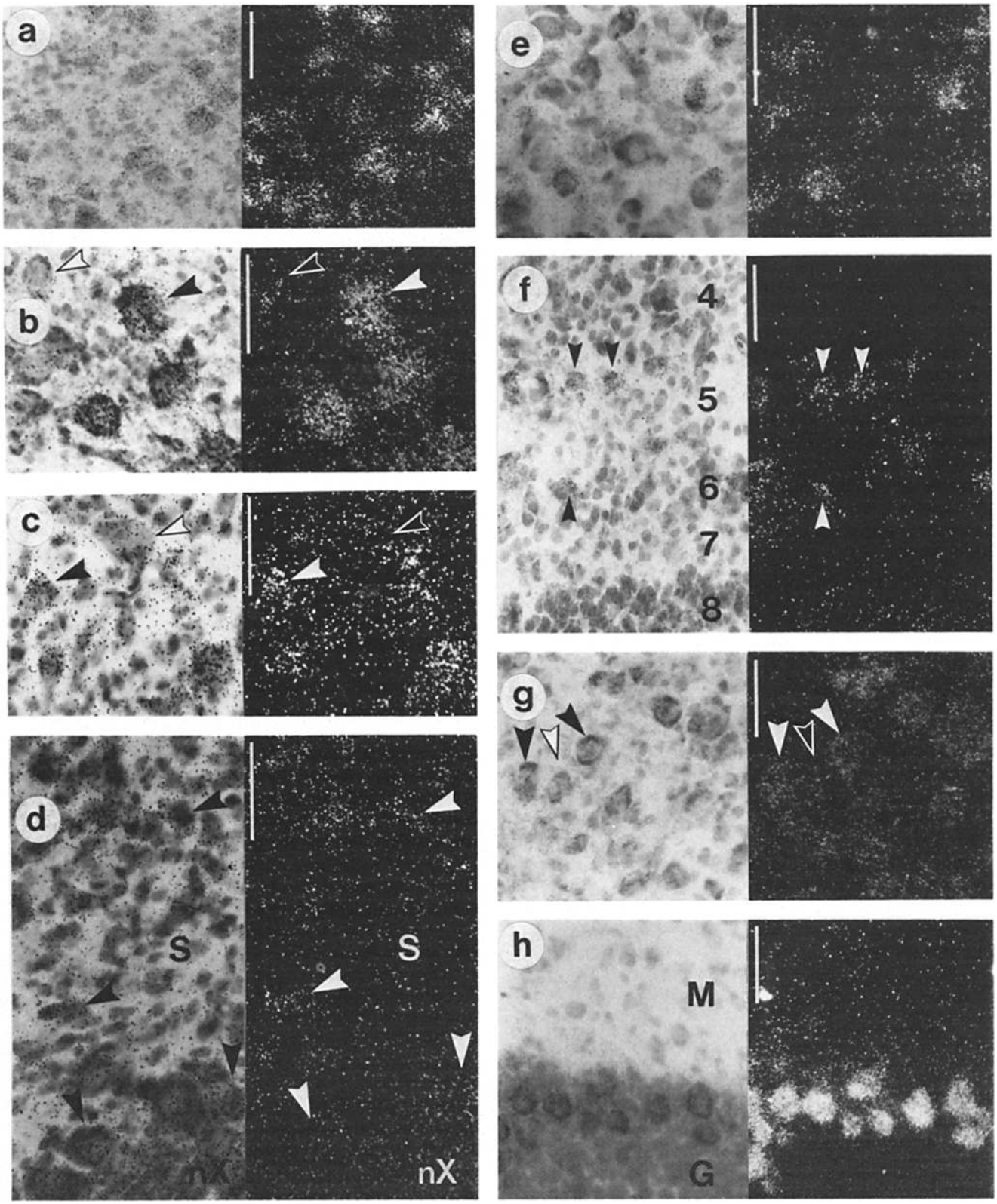


Figure 7. High power views of ISH to chick brain for calretinin (*a–d*) and calbindin (*e–h*). Each area is shown in bright field (showing histology and some silver grains) and dark field (showing only silver grains). Examples of positive cells are indicated with solid arrowheads, negative cells with open arrowheads. Calretinin: (*a*) Imc; (*b*) VeL; (*c*) reticular formation; (*d*) nuclei solitarius (*S*) and vagi (n*X*). All the large neurons in n*X* are positive. On similar sections, a calbindin probe lit up more cells in S and none in n*X*. Calbindin: (*e*) neostriatum; (*f*) tectum, layers 4–8; (*g*) nucleus rotundus (Rt); (*h*) cerebellum: *M*, molecular layer; *G*, granule layer. The Purkinje cells are strongly positive. Bars, 50 μm.

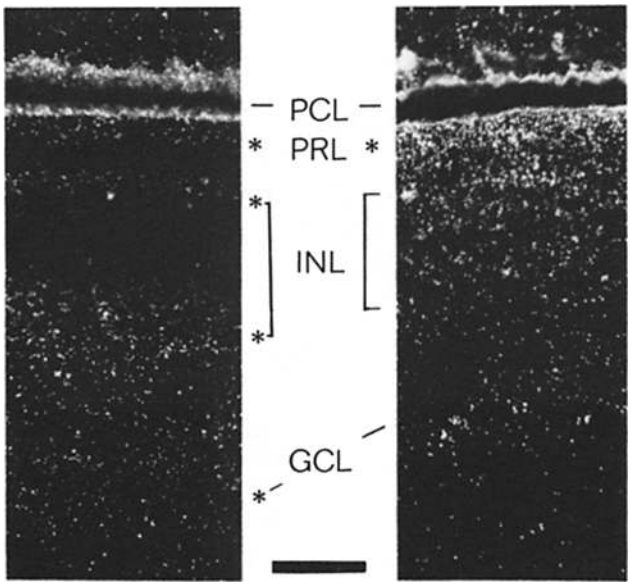


Figure 8. ISH to retina for calretinin (*left*) and calbindin (*right*). Dark-field views. *PCL*, pigment cell layer (which appears bright due to natural refringence); *PRL*, photoreceptor layer; *INL*, inner nuclear layer; *GCL*, ganglion cell layer. Asterisks indicate major positive layers. The calbindin section was printed at higher contrast and the signal apart from PRL was not always reproducible. Bar, 50 μm.

was absent from kidney, and a similar protein was detected in brains of chicks, rats, and frogs (49). It is probably identical to the calcium-binding protein of 31–33 kD seen in chick brain by Anthony and Babitch (3). In bovine brain, it could correspond to one of several calcium-binding proteins in the 21–27-kD range including caligulin (44, 67–69).

Function of CaBPs in Neurons

Calretinin is the third protein of this type found to be expressed in a restricted range of neurons, after calbindin and parvalbumin. It is the first for which neurons are the principal site of expression. All proteins of the superfamily are located in cytosol, and calbindin and parvalbumin immunoreactivities are detectable throughout many of the respective positive neurons, in dendrites and axons as well as cell bodies (5, 15, 29, 47, 54, 74). Although the antisera used by those authors did not detect immunoreactivity quite as extensively in the calretinin-containing neurons (e.g., nuclei isthmi magnocellularis, laminaris) as in the calbindin-containing neurons (e.g., cerebellum), this may be because of incomplete cross-reaction. In view of their close homology and mutually exclusive localization, it is likely that calbindin and calretinin perform similar functions in different neurons.

What this function might be is not yet known. Changes in calcium concentration are crucial to many dynamic processes in cells, and especially in neurons, where calcium is known to be involved in axonal transport, in releasing transmitters, and in transducing the responses to many stimuli. There are also gated calcium channels which can produce calcium action potentials in some neurons. The ubiquitous intracellular distribution of parvalbumin, calretinin, and calbindin would be consistent with a role in electrical transmission. This role could involve interacting with other proteins,

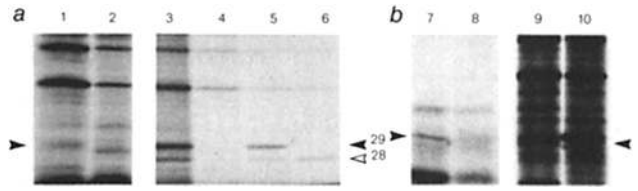


Figure 9. Identification of calretinin as a 29-kD polypeptide. (*a*) In vitro translation and immunoprecipitation. Poly(A)$^+$ RNA was translated in vitro and the products, with or without immunoprecipitation, were run on 9.5% SDS-PAGE. Lanes *1* and *2*, unfractionated products (short exposure): (lane *1*) retina; (lane *2*) midbrain (mesencephalon plus diencephalon minus optic lobes; cerebellum RNA gave a similar picture). (Lanes *3–6*) Products immunoprecipitated with antiserum against calbindin: (lane *3*) retina immunoprecipitate; (lane *4*) retina control (with normal rabbit serum instead of antiserum; controls on midbrain and cerebellum looked the same); (lane *5*) midbrain immunoprecipitate; (lane *6*) cerebellum immunoprecipitate. Calretinin (29 kD) is seen in lanes *1*, *3*, and *5*; calbindin (28 kD) in lanes *3*, *5*, and *6*. (*b*) Hybrid selection and in vitro translation. Products were run on 12% SDS-PAGE. (Lane *7*) Products from retina RNA hybrid-selected using calretinin cDNA; (lane *8*) control using unrelated DNA. Lanes *9* and *10*, as lanes *1* and *2*.

or facilitating calcium diffusion (10, 39), or providing short-term buffering for local calcium concentration in the cytosol. A calcium-buffering role in neurons has been suggested both for calbindin (5, 29, 30) and for parvalbumin (9, 13, 18), by analogy with evidence that they have calcium-buffering roles respectively in intestinal calcium absorption (60) and in muscle contraction (26, 48).

A possible function for such buffering may be to sharpen the timing of action potentials which depend in whole or in part on calcium fluxes, just as parvalbumin is thought to sharpen the timing of muscle contractions. Miller and Baimbridge (45) argued that such a function was consistent with the loss of calbindin immunoreactivity from the hippocampus of rats which have been rendered prone to seizures. Also, calbindin immunoreactivity is present in an electric fish, *Eigenmannia*, in all the neurons that fire in synchrony with the electric organ (11, 41). Calcium-buffering proteins could perhaps sharpen timing by shortening of action potentials in axons and shortening of transmitter release at presynaptic terminals.

Most relevant to the present study is the distribution of calbindin immunoreactivity in the auditory system of owls (12, 63). The primary auditory nuclei are angularis, which encodes sound intensity, and magnocellularis, which encodes timing. The magnocellularis neurons have action potentials locked to the phase of the sound waves and transmit bilaterally to nucleus laminaris, where timing differences as small as 10 μs between the two ears are measured to build up an

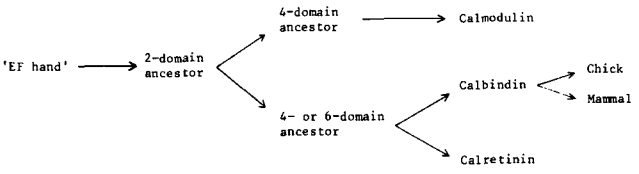


Figure 10. Evolution of calretinin as inferred from sequence comparisons.

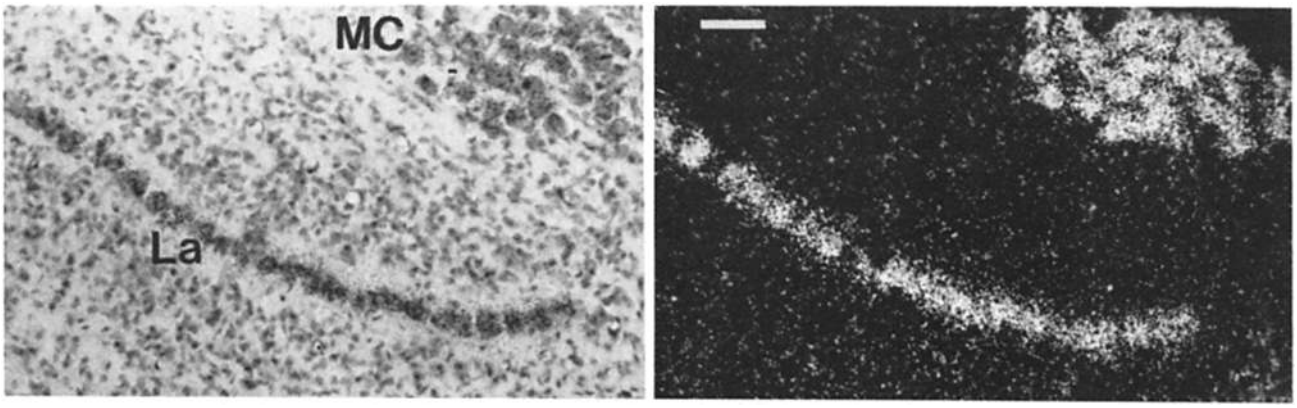


Figure 11. Calretinin ISH to auditory nuclei laminaris (*La*) and magnocellularis (*MC*): bright-field and dark-field views. This area contained no calbindin RNA. Bar, 50 μm.

auditory map of space (35, 61). The neurons of nuclei magnocellularis and laminaris have high levels of calbindin immunoreactivity in owls, as in chicks (29), and this was suggested to be responsible for the temporal precision of their responses (12, 63). The present study shows that this immunoreactivity is in fact all due to calretinin. The auditory nuclei angularis, magnocellularis, and laminaris are among the most concentrated locations of calretinin RNA in the chick brain (Fig. 11). The nuclei further along the auditory pathway—superior olive, ventralis lemnisci lateralis, and inferior colliculus—contained immunoreactive fibers but not somata (63), and ISH confirms that they are largely negative for calretinin mRNA. Thus the preservation of phase-locked action potentials as far as the third neurons (in laminaris) of the auditory pathway may require the presence of calretinin.

In summary, calbindin and calretinin are generally expressed in different neurons throughout the brain, and many of these neurons are notable for the precise timing of their discharges. Similarly, parvalbumin is generally expressed in a third set of neurons, which share the property of rapid firing rate (9, 14). It is hoped that more precise definition of the various neurons expressing these calcium-binding proteins, and studies of the ionic fluxes in these neurons, will show whether these suggestions for functions are correct, and why three similar, though evolutionarily distinct, calcium-binding proteins should be expressed in different sets of neurons.

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