Expression of the calsequestrin gene in chicken cerebellum Purkinje neurons

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Intracellular rapidly exchanging Ca^{2+} stores are identified and defined in terms of intralumenal low-affinity, high-capacity Ca^{2+}-binding proteins, of which calsequestrin (CS) is the prototype in striated muscles. In chicken striated muscles, there is a single gene for CS [Choi and Clegg (1990) Dev. Biol. **142**, 169–177]. In the chicken brain, the gene for CS was found to be selectively expressed in Purkinje neurons, as judged by Northern blotting, *in situ* hybridization and immunocytochemistry. The synthetic machinery for CS was found to be restricted to the cell body, i.e. excluded from dendrites and axon.

INTRODUCTION

Redistribution of Ca^{2+} from intracellular rapidly exchanging Ca^{2+} stores plays a key role in several functions of eukaryotic cells. The molecular components predicted to be present in Ca^{2+} stores are Ca^{2+}-release channels, Ca^{2+} pumps and intralumenal low-affinity, high-capacity, Ca^{2+}-binding proteins [1], for release, uptake and storage respectively of Ca^{2+}.

The identification of intralumenal Ca^{2+}-binding proteins has received much attention in recent years. It appears that there are at least two structurally distinct classes of low-affinity, high-capacity, Ca^{2+}-binding proteins: the calsequestrins (CSs [2–5]) and the calreticulins [6–8]. CS appears to be expressed in striated muscles, probably in smooth muscles (cf. [9] and [10]), and, among non-muscle cells, in chicken Purkinje neurons [11–13]. In chicken striated muscle there appears to be a single gene for CS [14,15], whereas in mammalian striated muscle there are two different genes [2,3] encoding two CS isoforms, one for cardiac muscle and one for fast-twitch skeletal muscle.

In this paper, we provide evidence for the transcription of the CS gene in chicken Purkinje neurons by using Northern blot and *in situ* hybridization experiments. The synthetic machinery for CS was found to be compartmentalized, i.e. restricted to the cell body and excluded from the dendrites and axon. The latter finding has relevance for the mechanisms of intracellular trafficking of CS, a protein that is distributed not only to the cell body but also to the dendrites, axon and axon terminal [13].

MATERIALS AND METHODS

Tissue sources

The pectoralis major muscle, cardiac ventricle, liver, cerebrum and cerebellum were quickly removed from 2-month-old chickens that had been killed by decapitation and either immediately frozen in liquid nitrogen for preparation of total RNA or fixed with 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization.

DNA and RNA probes

The full-length cDNA clone of chicken skeletal muscle CS has been described previously [14,15]. The *Bam*HI–*Eco*RI probe used in both Northern blotting and *in situ* hybridization corresponds to the first 406 nucleotides of the 5′-translated region of the CS cDNA.

cDNA probes for Northern blotting were ^{32}P-labelled by random priming [16].

Sense and antisense cRNA probes for *in situ* hybridization were transcribed *in vitro* and labelled with [^{35}S]UTP [17]. Each probe was digested to 50–100 nt fragments by mild alkaline hydrolysis.

Northern blot analysis

Total RNA was extracted from chicken tissues [18], electrophoresed on denaturating 1% agarose gels and transferred to nylon membranes [19,20]. Hybridization was carried out overnight at 65 °C with ^{32}P-labelled cDNA probes in 1 mM EDTA, 20 mM sodium phosphate buffer, pH 7.2, 100 mM NaCl, 50 mM Pipes, pH 6.8, and 5% SDS. Washes were carried out at 65 °C: four 30 min washes in 1 × SSC, 5% SDS and 10 mM sodium phosphate buffer, pH 7.2, were followed by three 15 min washes in 0.5 × SSC, 0.1% SDS, 10 mM sodium phosphate buffer, pH 7.2.

In situ hybridization

In situ hybridization was performed on either 5 μm-thick paraffin sections (see Figure 3b) or 10 μm-thick cryostat sections, as described [17]. Prehybridization was carried out at room temperature and included fixation for 30 min with 4% paraformaldehyde in PBS, digestion for 7.5 min with proteinase K in 10 mM Tris/HCl and 1 mM EDTA, pH 7.8, and treatment for 10 min with 0.25% acetic anydride/0.1 M triethanolamine. Sections were dehydrated and hybridized overnight at 52 °C to 5×10^5 c.p.m. of probe and then washed at 65 °C in 50% formamide, 2 × SSC and 0.1 M dithiothreitol. Slides were processed for autoradiography using Kodak NTB-2 emulsion and exposed for 7–21 days. A Zeiss Axioplan microscope equipped with dark-field and phase-contrast optics was used for examination. Sections serial to those processed for *in situ* hybridization were also immunostained with anti-CS antibodies (see below).

Abbreviations used: CS, calsequestrin; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SSC, saline sodium citrate (0.15 M NaCl/0.015 M sodium citrate).

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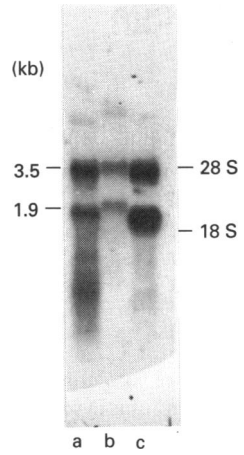

Figure 1 Northern blot analysis of CS mRNA

Samples of 30 μg of total RNA obtained from chicken cardiac ventricle (lane a), cerebellum (lane b) and skeletal muscle (lane c) were hybridized with the ^{32}P-labelled cDNA probe. Hybridization and washes were carried out as described in the Materials and methods section.

Immunocytochemistry

Cerebellum was fixed in 4% paraformaldehyde/0.25% glutaraldehyde and processed as previously described [12]. Cerebellum cortex parasagittal sections were stained by immunofluorescence with anti-(chicken skeletal muscle CS) antibodies [5,11] using rhodamine-conjugated anti-rabbit IgGs.

RESULTS AND DISCUSSION

Northern blot analysis

In a first series of experiments, we examined the transcription of the CS gene in several different tissues in the chicken. Northern blot analysis of total RNA with the 406 bp probe of the 5′-translated region revealed two hybridizing RNA species in cardiac ventricle, cerebellum and skeletal muscle (Figure 1), whereas cerebrum and liver samples showed no hybridization (results not shown). In muscles (lanes a and c), the mRNA band of 1.9 kb, corresponding in size to the cloned CS cDNA [14], and the band of 3.5 kb might arise from differential use of two polyadenylation sites [3,14,15]. Interestingly, as shown in Figure 1 (lane b), the cerebellum mRNA band of about 2 kb appeared to have slower electrophoretic mobility, i.e. a slightly larger size,

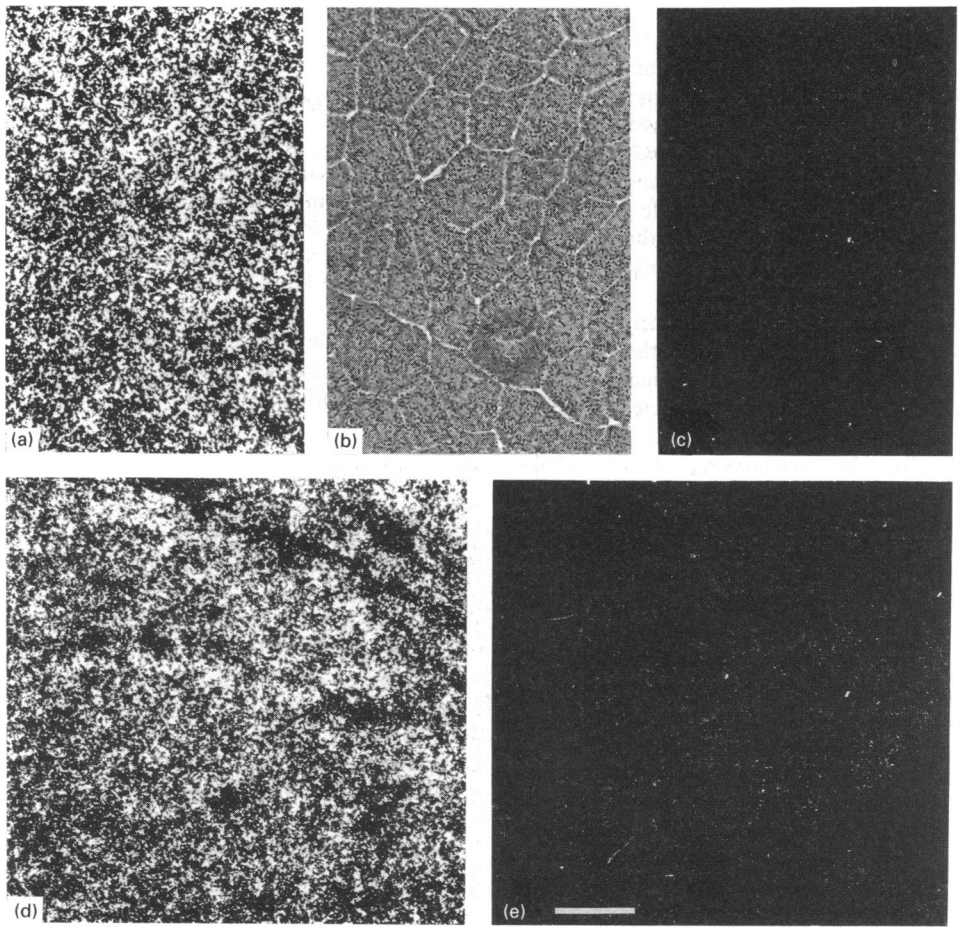

Figure 2 *In situ* hybridization of chicken skeletal muscle and heart

Dark-field photomicrographs are shown of serial cryosections of skeletal muscle (panels **a** and **c**) and of cardiac ventricle (panels **d** and **e**) hybridized either with the CS cRNA antisense probe (**a** and **d**) or with the CS cRNA sense probe (**c** and **e**). Panel (**b**) shows a phase-contrast image of the skeletal muscle transverse section. Bar, 60 μm.

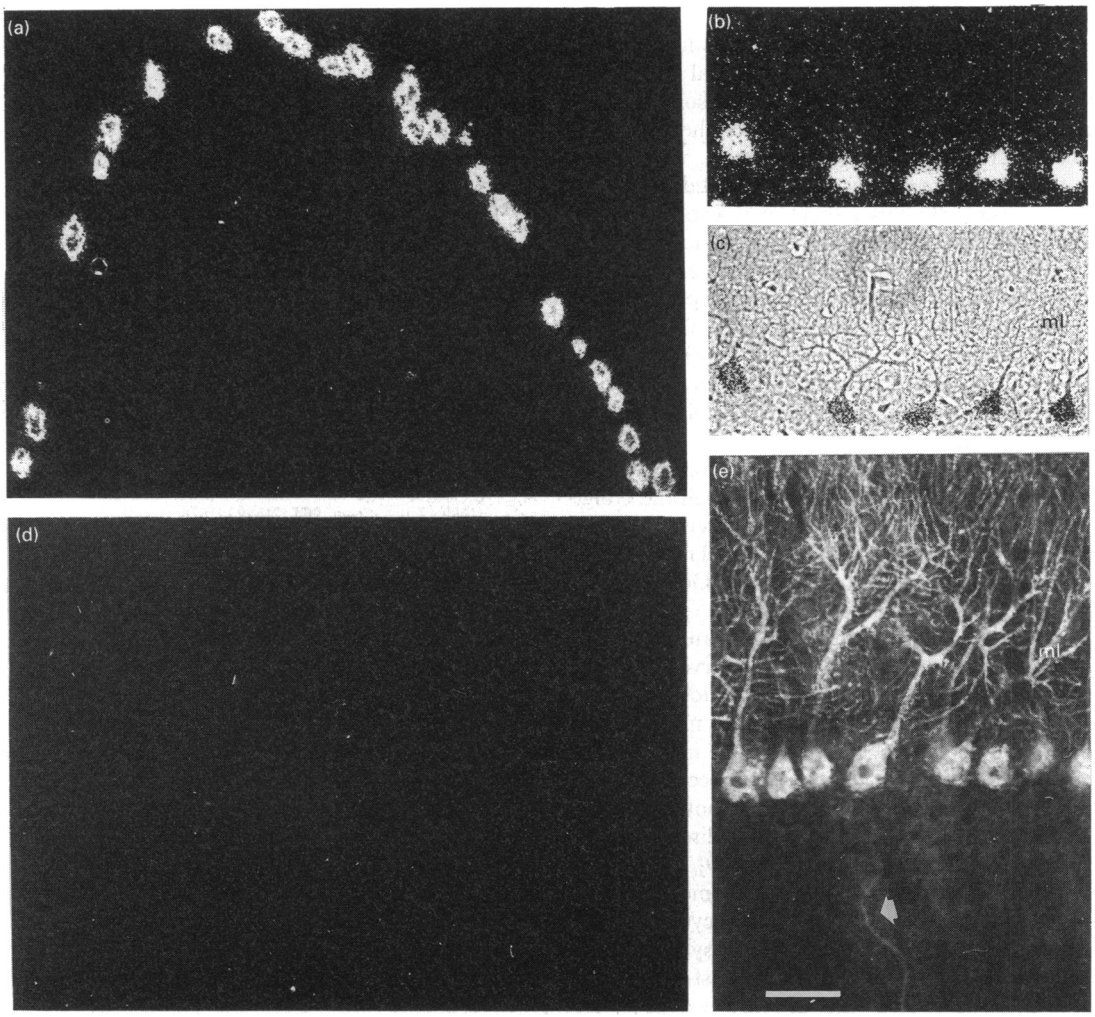

Figure 3 ***In situ*** **hybridization and immunofluorescence of chicken cerebellum cortex**

Dark-field photomicrographs are shown of parasagittal sections (panels **a**, **b** and **d**) hybridized with either the CS cRNA antisense probe (panels **a** and **b**) or the CS cRNA sense probe (panel **d**). (**c**) Phase-contrast image of the same section shown in (**b**); (**e**) immunofluorescence labelling of a cerebellum cortex section with anti-CS antibodies; the soma of Purkinje neurons, dendrites in the molecular layer (ml) and axons (arrow) are labelled. Bar: (**a**) and (**d**), 120 μm; (**b**) and (**c**), 60 μm; (**e**), 40 μm.

than the cardiac and skeletal muscle bands thus suggesting the presence of different RNA transcripts. This possibility, however, was not investigated further.

In situ hybridization

The transcription of the CS gene was further investigated at the mRNA level by *in situ* hybridization (Figures 2 and 3). Microscopic examination of cryosections hybridized with the antisense cRNA probe revealed intense and homogeneous labelling of both skeletal muscle fibres (Figure 2a) and cardiac ventricle myocytes (Figure 2d). Figure 2(b) shows the phase-contrast image of the skeletal muscle section. Hybridization with the sense cRNA probe was negative in skeletal muscle and heart (Figures 2c and 2e).

In parasagittal sections of the cerebellum cortex (Figures 3a and 3b), only the soma of Purkinje neurons were intensely labelled by the antisense cRNA probe, whereas dendrites and, as expected, axons were not labelled. Phase-contrast examination (Figure 3c) of the same section shown in Figure 3(b), as well as immunofluorescent decoration with anti-CS antibodies of serial sections (results not shown), confirmed the presence of dendrites in the molecular layer (labelled ml in Figure 3c). No hybridizing signal was detected either in other cerebellum neurons or in the cerebrum (results not shown). Hybridization of cerebellum sections with the sense cRNA probe (Figure 3d) was not observed.

We have thus provided evidence for the selective transcription of the CS gene in the Purkinje neurons of chicken cerebellum.

Compartmentalization of the synthetic machinery for CS

As shown by immunofluorescence studies of parasagittal sections of fixed cerebellum cortex, CS is present in the soma of Purkinje neurons, dendritic trees spreading into the molecular layer and axons (Figure 3e; see also [12,13]). As also shown by electron microscope immunocytochemistry, CS accumulates in specialized domains of the sarcoplasmic reticulum (SR) of both skeletal muscle (junctional cisternae [21]) and heart (junctional cisternae and corbular SR; [22]), and in moderately dense-cored vacuoles of 100–150 μm in diameter ('calciosomes') in chicken Purkinje

neurons [11–13]. Calciosomes are widely distributed in the soma, dendrites, axon and axon terminal, but not in dendritic spines [12,13]. Some calciosomes appear to be in lumenal continuity with the smooth endoplasmic reticulum (ER), but some appear to be disconnected from the ER network, i.e. they may be discrete organelles [12,13].

In contrast, CS mRNAs are clearly compartmentalized in the soma of Purkinje neurons. (In this respect, it is known that in hyppocampal and sympathetic neurons in culture, mRNAs for some proteins, such as α- and β-tubulins, GAP-43 and NF 68, are confined to the cell body whereas the encoded proteins are also present in the dendrites and axons [23,24]). The present observations lead to questions concerning the mechanisms of spatial restriction of the synthetic machinery for CS on the one hand, and the intracellular trafficking of CS and the biogenesis of CS-enriched compartments on the other.

The differential distribution of the synthetic machinery for CS is likely to be related to the processing of CS through the Golgi complex, a key feature of CS maturation that occurs in both muscle fibres and Purkinje neurons. In chick skeletal muscle [25], CS has been reported to travel through the cis-Golgi before reaching its final destination, the junctional SR. In chicken Purkinje neurons [12], CS has been detected in lateral tips of Golgi cisternae and direct continuity has been observed between the latter cisternae and some of the juxta-Golgi calciosomes. It is relevant to note that, unlike the mRNAs for CS, mRNAs for other membrane-bound molecular components of intracellular Ca^{2+} stores, e.g. the Ca^{2+} pump [26] and the $InsP_3$ receptor/Ca^{2+}-release channel [27,28], appear to be distributed in both the soma and the dendrites. The specific mechanisms that direct mRNA localization remain, however, to be determined [29].

The relationship between calciosomes and the smooth ER is unclear at the present time, particularly in the cytoplasm of peripheral dendrites and axon terminals. Since the synthesis and processing of CS are confined to the soma, post-Golgi CS-enriched vacuoles may be routed into at least two potential pathways. (a) They may fuse back with the ER of the soma and discharge CS into the ER lumen (connections between the cis-Golgi and the smooth ER have been described in neurons [30]); CS would then diffuse intralumenally, move outwards and concentrate at specific membrane sites endowed with anchoring CS-binding protein(s), as well as with other molecular components of Ca^{2+} stores [11–13,26]. Eventually, CS-enriched domains could either define specialized ER subcompartments or bud off and become discrete organelles [13]. (b) Post-Golgi CS-enriched vacuoles may be carried outwards by fast axonal transport, in either dendrites or axons, and fuse back with elements of the smooth ER. Additional molecular components of Ca^{2+} stores would reach their final destination by lateral diffusion and/or by local synthesis and co-translational membrane insertion. At the end of this pathway, CS-enriched domains could either define specialized ER subcompartments or bud off and become discrete organelles [13]. Additional experiments are needed to distinguish between the two proposed pathways.

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