Molecular cloning of frog secretogranin II reveals the occurrence of several highly conserved potential regulatory peptides

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Received 12 July 1996; revised version received 27 August 1996

Abstract Secretogranin II (SgII) is an acidic secretory protein present in large dense core vesicles of neuronal and endocrine cells. Based on the sequence of a peptide derived from the processing of SgII in the brain of the frog *Rana ridibunda*, degenerate oligonucleotides were used to clone the cDNA encoding frog SgII from a pituitary cDNA library. This cDNA encodes a 574 amino acid protein which exhibits 46–48% sequence identity with mammalian SgII and contains 11 pairs of basic amino acids. Four potential processing products delimited by pairs of basic residues exhibited a much higher degree of identity (68–82%) with the corresponding mammalian SgII sequences. The frog SgII mRNA is ~4 kb in length and is differentially expressed in the brain and endocrine tissues. The present data reveal that several SgII-derived peptides have been highly conserved during evolution, suggesting that these peptides may play important neuroendocrine regulatory functions.

Key words: Secretogranin II; Chromogranin; Secretoneurin; Peptide precursor; Frog

1. Introduction

Secretogranin II (SgII) is a member of a family of acidic secretory proteins called 'granins' which also includes chromogranin A (CgA) and chromogranin B (CgB) [1]. Granins are characterized by their high content of acidic amino acids, their localization in secretory organelles of endocrine cells and neurons, and the presence in their sequences of multiple pairs of basic residues which represent potential cleavage sites for processing enzymes [2,3].

In addition to a proposed role in the formation of secretory granules in neuronal and endocrine cells, the granins may serve as precursors for biologically active peptides [1–3]. In particular, SgII contains nine pairs of basic amino acids in all mammalian species studied so far, some of which are actually cleaved to generate shorter peptides [4,5]. In fact, several peptides derived from the proteolytic processing of SgII have already been isolated and characterized from the brain or endocrine tissues [6–8]. For instance, we have previously identified a 33-amino acid peptide arising from the proteolytic cleavage of SgII from the brain of the frog *Rana ridibunda* [6]. A homologous peptide, named secretoneurin (SN), has been subsequently identified in the brain and various endocrine tissues in mammals [9]. Functional studies revealed that SN stimulates dopamine release in the rat striatum [10,11] and triggers migration of human monocytes in vitro [12] thus establishing SN as an authentic regulatory neuropeptide.

Although SN has been initially isolated from the frog brain, the primary structure of frog SgII has not been characterized and therefore the phylogenetic conservation of this granin throughout vertebrates is still unknown. We now report the cloning of the cDNA encoding SgII in the frog *Rana ridibunda* and its expression in the brain and endocrine tissues.

2. Materials and methods

2.1. PCR, cloning and sequencing

A frog (*Rana ridibunda*) pituitary cDNA library (9.6×10^6 recombinants) was constructed in the plasmid CDM7/amp (T.B. Usdin, Laboratory of Cell Biology, NIMH, NIH) [13] and used in a PCR reaction to amplify the DNA fragment corresponding to the frog SN sequence. The PCR was carried out in a 50-μl volume containing 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% Tween 20, 200 μM of each dNTP, 1 μM of degenerate sense primer FSG1, 5′-AA(C/T)GA(A/G)AT(A/T/C)GT(A/G/C/T)GA(A/G)GG-3′ and antisense primer FSG2, 5′-TT(A/G)TT(G/A/T)GC(C/T)TG(G/T/C)CC(C/T)TT-3′ corresponding respectively to the hexapeptide sequences NEIVEG and KGQANN of the frog SN extremities [6], 240 ng of the frog pituitary cDNA library and 2.5 units Taq DNA polymerase (Eurobio) in a Robocycler Gradient 40 (Stratagene). After 4 min of denaturation at 94°C, 45 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 40°C for 1.5 min and extension at 72°C for 1.5 min, followed by an additional extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1.8% agarose gel and two bands with a size ≤125 bp (corresponding to the lower size marker used) were purified and ligated into the pGEMT vector (Promega). Sequencing showed that one of these bands corresponded to the DNA sequence encoding frog SN. This sequence was used to design a homologous antisense oligonucleotide, SN1, 5′-GAGTTCCTGGAAGACAGACTGTAAGGTGGCTAGGCTCTGAGGAGTATA-3′, which was used as a primer along with a T7 primer (present in the cDNA library vector) in a second PCR reaction in order to amplify the 5′ region of frog SgII from the library. The PCR conditions were as above, except that 1.5 mM $MgCl_2$ was used and 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 2 min were performed. The DNA fragment obtained, RFSN3, was inserted into the pGEMT vector and partially sequenced to verify that it actually corresponded to the SgII cDNA sequence.

2.2. Cloning of the full-length frog SgII cDNA

The DNA fragment RFSN3 was labeled by random-priming (Oncor) and used to screen 10^5 recombinants of the frog cDNA library. Several positive clones were obtained and their inserts were characterized by restriction mapping and Southern blotting analysis. Restriction fragments of the positive clone FSG82 with a ~3.8 kb insert were subcloned into pBluescript II KS (Stratagene) and sequenced on

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Abbreviations: CgA, chromogranin A; CgB, chromogranin B; PCR, polymerase chain reaction; 5′ RACE, rapid amplification of cDNA 5′ end; SgII, secretogranin II; SN, secretoneurin

The sequence reported in this paper has been deposited in the GenBank data base (database no. U68757).

PII S0014-5793(96)00976-3

both strands with T7, T3 and internal primers using the Sequenase kit (Amersham). The junctions between the fragments were confirmed by sequencing the corresponding regions on the full-length clone FSG82 using internal primers.

2.3. Northern blot analysis

Total RNA from frog tissues was prepared by the acid guanidinium thiocyanate-phenol-chloroform method [14] using the Trizol reagent (Life Technologies). Equivalent amounts of RNA (20 μg) were analyzed as described previously [15] and hybridized to ^{32}P-labeled random-primed RFSN3 containing the 5′ region of frog SgII. Hybridization was performed at 42°C and the blot washed at 50°C in standard conditions [16].

2.4. In situ histochemical hybridization

Frogs were anesthetized and perfused transcardially with 4% paraformaldehyde. Frontal sections (12 μm) of post-fixed and embedded brains were cut in a cryostat and kept at −80°C until use. Tissue sections were hybridized at 60°C in the presence of 10^7 cpm/ml ^{35}S-labeled riboprobe as previously described [17]. Sense and antisense riboprobes were prepared by in vitro transcription of an *Eco*RI-*Hin*dIII fragment (containing bases 1–738 of FSG82) subcloned into pBluescript II KS, in the presence of [^{35}S]UTP (Amersham) and T7 or T3 RNA polymerase [18]. After hybridization, tissue slices were dehydrated and exposed onto Hyperfilm β max (Amersham) for 2 days. The slides were subsequently dipped into Kodak NTB-2 liquid emulsion at 40°C, exposed for 15 days and developed. In order to identify anatomical structures, the brain sections were stained with hematoxylin and eosin.

3. Results and discussion

3.1. Cloning and characterization of the frog SgII cDNA

Since in mammals the SgII gene is actively expressed in the pituitary [19], the cloning of the frog SgII cDNA was performed using a frog pituitary library, even though frog SN was initially characterized from the frog brain [6]. Degenerate oligonucleotides deduced from the sequence of frog SN were used to amplify by PCR the cognate DNA sequence from a *Rana ridibunda* pituitary library. A product of the PCR reaction gave the expected sequence encoding the frog SN peptide (data not shown). This sequence was then used to synthesize a homologous oligonucleotide in order to amplify the 5′ region of the frog SgII cDNA from the library by means of a 5′ RACE reaction (data not shown). The PCR product (∼600 bp) was used as a probe to screen the library for full-length SgII cDNA clones. This approach was chosen rather than screening the library with the homologous oligonucleotide in order to increase the probability of isolating full-length 5′ region-containing clones. Screening of the cDNA library with the 5′ RACE product led to the isolation of several clones with inserts ranging between 1.6 and 3.8 kb. The large size of the majority of the clones isolated was intriguing inasmuch as the mammalian SgII encoding mRNA is ∼2.5 kb in length [2]. Sequencing of the longest clone revealed an open reading frame of 1803 bp encoding a 601 amino acid protein. Most of the extra sequences lay in the 3′-untranslated region of the cDNA (Fig. 1 and data not shown). The first 27 amino acids of the encoded protein correspond to a putative signal peptide based on the conservation of the cleavage site found in mammalian SgII [20], and the remaining 574 amino acids represent the mature frog SgII protein with a deduced molecular weight of 67 001 Da. This protein contains a putative tyrosine sulfation site at position 124 surrounded by acidic amino acids homologous to the tyrosine sulfation site found in mammalian SgII [2], and a potential *N*-glycosylation site at position 280 (Fig. 1).

Another important structural feature shared with mammalian SgII is the high content of acidic amino acids in the frog protein with 20% of its residues being either aspartic acids or glutamic acids. The high proportion of acidic residues suggests that frog SgII, like its mammalian counterpart [21], may play a role in the formation of secretory granules and/or in the packaging of their resident neuropeptides and peptide hormones [1–3].

3.2. SgII as a propeptide protein

Characterization of the primary structure of SgII in mam-

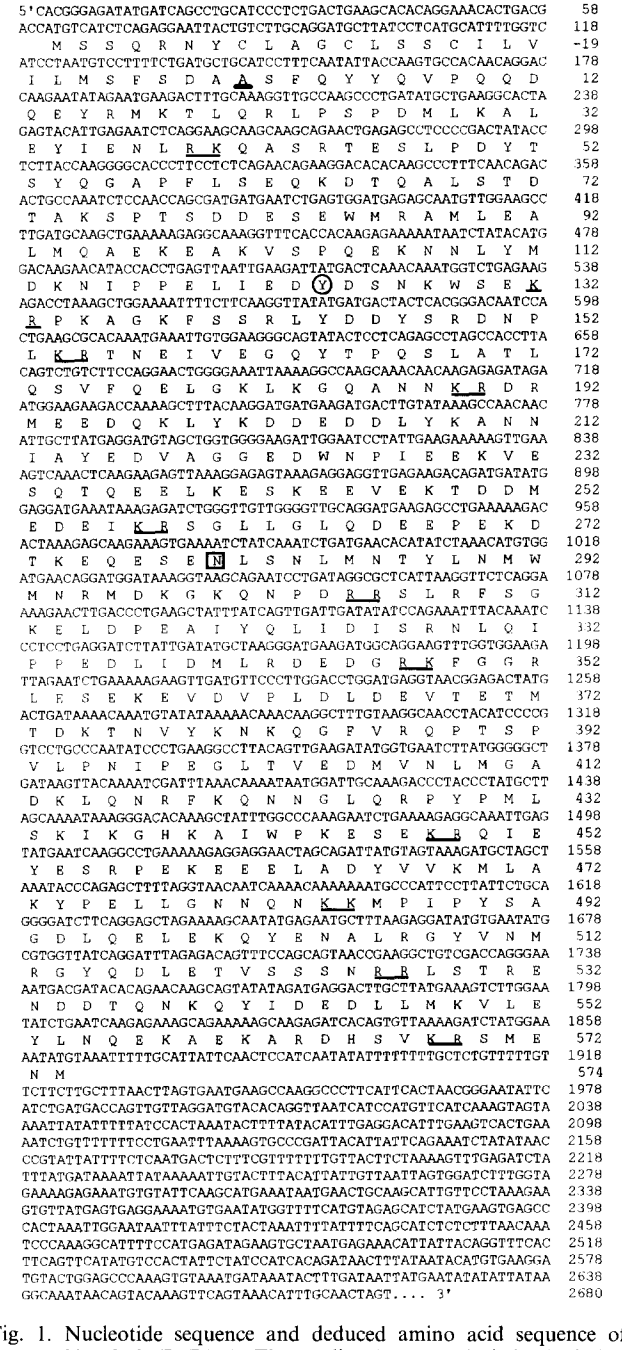

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Rana ridibunda* SgII cDNA. The predicted preprotein is in single letter amino acid code and is numbered on the right along with the nucleotide sequence. The N-terminal Ala residue of the mature protein is underlined in bold. The 11 pairs of basic amino acids are underlined, the potential sulfation site is circled and the potential *N*-glycosylation site is boxed.

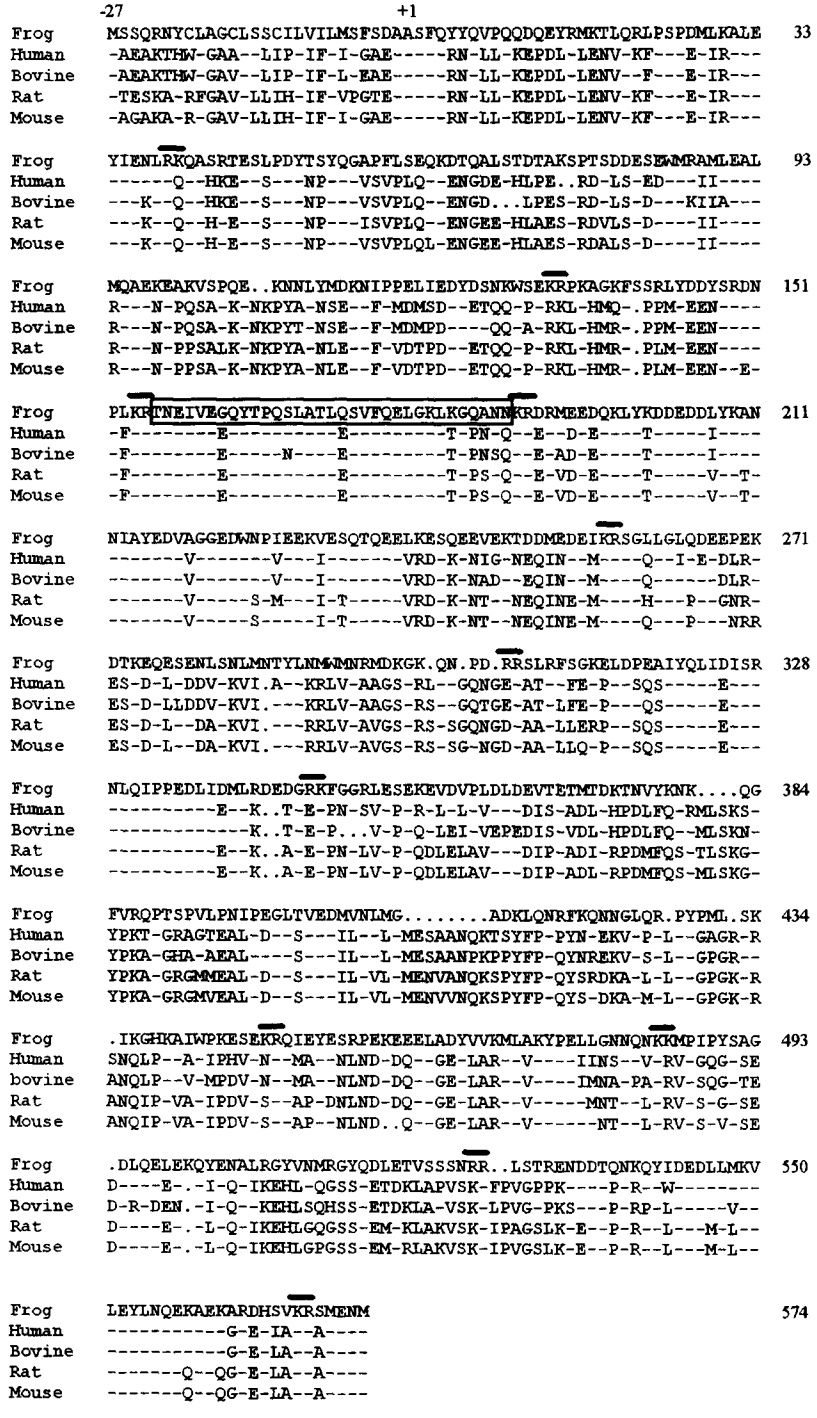

Fig. 2. Alignment of the amino acid sequence of frog SgII with those of human [21], bovine [20], rat [27] and mouse [28] SgII. Identical amino acids between the frog SgII sequence and the other sequences are indicated by dashes. The dots in the sequences indicate gaps introduced for the alignment purpose. The numbers on the right correspond to the frog SgII amino acids. The bold lines above the frog SgII sequence indicate the dibasic sites and the boxed sequence corresponds to frog secretoneurin [6].

mals has revealed the occurrence of nine pairs of basic amino acids [1,2] which can be processed in vivo to generate bioactive peptides such as SN [9]. Frog SgII contains eleven pairs of basic amino acids (Fig. 1), seven of which are common to mammalian and frog SgII. The additional pairs of basic residues found in frog SgII correspond to single basic amino acids in the mammalian sequences (Fig. 2). The alignment of the frog and mammalian proteins shows an overall identity of 46–48%, several regions being more conserved than others. In particular, four peptide sequences delimited by conserved dibasic cleavage sites possess a high degree of structural identity (Fig. 3). Among these peptides, SN (denoted by number II in Fig. 3) exhibited the highest sequence conservation across species (82% identity between the frog and human peptides). Previous studies have shown that SN is actually processed in the frog [6] and bovine brain [9] and this peptide has been found to stimulate dopamine release from the rat striatum [10,11]. Three other putative peptides denoted by numbers III, V and VI in Fig. 3 also exhibited a high degree of sequence conservation (68, 70% and 80% identity between the

frog and human SgII sequences, respectively). The rat counterpart of peptide number V, named LA-42, has been previously isolated from pituitary gonadotroph-conditioned medium [7] while the bovine counterpart of peptide number VI has been characterized from purified secretory granules of adrenal chromaffin cells [8]. It has also been found that this latter peptide was released from cultured bovine chromaffin cells, together with catecholamines, upon nicotinic stimulation [8]. The strong conservation of these four peptides from amphibia to mammals and the fact that three of them have already been characterized in tissue extracts and/or culture media support the concept that SgII may serve as a precursor for several regulatory peptides. In contrast, two other peptides (numbered I and IV in Fig. 3) are unlikely to occur as free regulatory peptides since their sequences are poorly conserved. In fact, there is evidence that peptide number I is not formed in vivo [9,22].

3.3. Distribution of the frog SgII mRNA

Expression of the frog SgII gene in different tissues was studied by Northern blot analysis. A strong hybridization signal corresponding to an mRNA of ~4 kb was observed in the brain, hypothalamus, pituitary and spinal cord. In the adrenal gland, the concentration of SgII mRNA was much lower (Fig. 4). No signal could be detected in liver, spleen or testis (data not shown). These data confirmed the larger size of the frog SgII mRNA (~4 kb) compared to mammalian SgII mRNA (2.5 kb) and the neuronal- and endocrine-specific expression of the frog SgII gene as previously reported in mammals [2]. The molecular mechanism underlying the restricted expression of the SgII gene is still unknown. However, a functional cAMP-responsive element has been recently characterized in the mouse SgII promoter [23]. This *cis*-regulatory element might also confer, alone or in combination with other *cis*-acting element(s), tissue-specific expression to the SgII gene as was shown for another member of the granin family, CgA [24,25].

In situ hybridization histochemistry revealed the widespread distribution of the SgII mRNA in the frog brain. In the telencephalon, prominent hybridization signal was found in the pallium and the amygdaloid complex (Fig. 5A). In the diencephalon, a strong signal was observed in the anterior preoptic area, the dorsal and ventral hypothalamic nuclei and the posterior thalamic nucleus (Fig. 5A,B). In contrast, other regions such as the medial septum, the lateral thalamic nucleus and the optic tectum only showed a low hybridization signal (Fig. 5A,B). At a high magnification, it appeared that, in the brain regions which exhibited intense labeling, several cells strongly expressed the SgII gene while other cells were totally

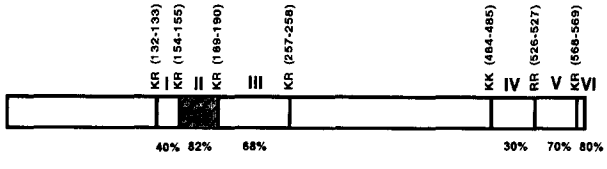

Fig. 3. Schematic representation of the structure of SgII showing the conservation of processing sites and sequences between frog and human SgII. The numbers in parenthesis refer to the position of the pairs of basic amino acids and the roman numbers designate the peptides which can be potentially generated from the processing of these dibasic sites. The hatched zone corresponds to secretoneurin [6,9]. The percentage of amino acid identity between the frog and human sequences are indicated under each putative peptide.

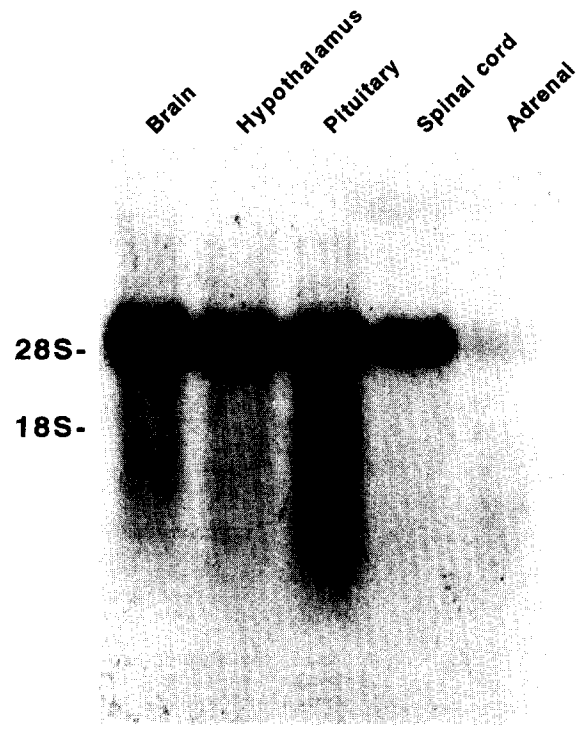

Fig. 4. Northern blot analysis of frog SgII mRNA. Approximately the same amounts of total RNA (20 μg) from different tissues were hybridized to the ^{32}P-labeled random-primed RFSN3 probe and exposed to X-AR5 film for 10 days with intensifying screens. Ribosomal 28S and 18S RNA positions are indicated in the left margin.

devoid of SgII mRNA (Fig. 5C,D). The differential expression of the SgII gene in various regions of the brain as previously reported in rat [26] and in discrete cells within each region, strongly suggests that SgII and/or its processing products may play an important role in the regulation of neuronal activity.

In conclusion, the characterization of the frog SgII cDNA has revealed the existence of four peptide sequences, in the SgII protein, which have been highly conserved during evolution. These data, together with the differential expression of the SgII gene in the brain and endocrine tissues, support the view that SgII may serve as a precursor for biologically active peptides.

3.4. Note added in proof

Holthuis and Martens [30] have recently described the cloning of a cDNA encoding the SgII of *Xenopus laevis*, which also exhibits a high regional conservation with the mammalian SgII.

Acknowledgements: This work was supported by grants from INSERM (U413) and the Conseil Régional de Haute-Normandie. We thank Dr. T.B. Usdin (Lab. Cell Biology, NIMH, NIH) for the preparation of the frog pituitary library and P. Bizet for excellent technical assistance. Y.A. was a recipient of a postdoctoral fellowship from the Conseil Régional de Haute-Normandie.

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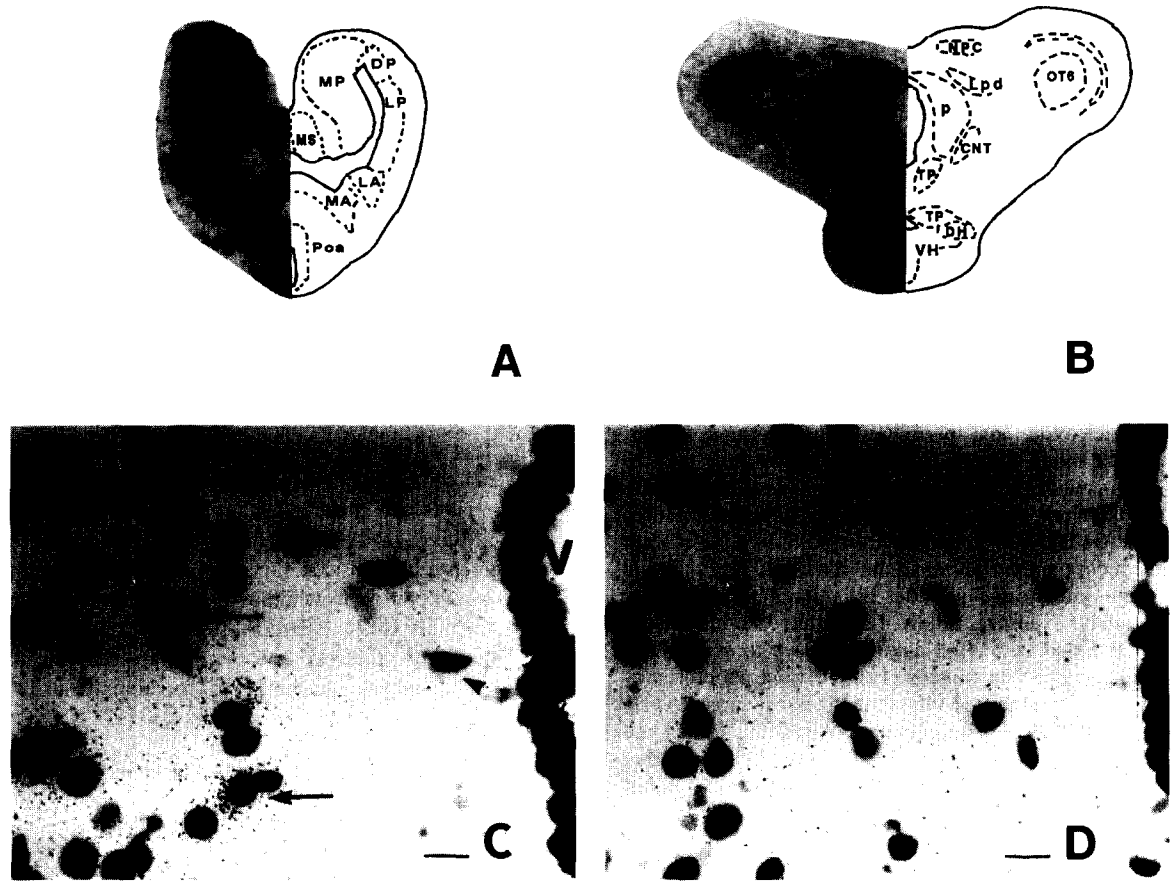

Fig. 5. In situ hybridization of SgII mRNA in the brain of *Rana ridibunda*. A, B: Autoradiographs showing the distribution of SgII mRNA in frontal frog brain hemisections at the level of the telencephalon (A) and diencephalon (B). Exposure time, 2 days. The anatomical structures, adapted from the atlas of Neavy and Northcutt [29], are designated on the right hemisection. C, D: Photomicrographs of SgII mRNA containing cells in the periventricular part of the thalamus. Hybridization was carried out with antisense (C) or sense (D) probes. Exposure time, 15 days. The arrows indicate strongly hybridizing cells and the arrowheads show unlabelled cells. Abbreviations: CNT, central thalamic nucleus; DH, dorsal hypothalamic nucleus; DP, dorsal pallium; LA, lateral amygadala; LP, lateral pallium; Lpd, lateral thalamic nucleus; MA, medial amygdala; MP, Medial pallium; MS, medial septum; OT6, optic tectum (lamina 6); Poa, anterior preoptic area; P, posterior thalamic nucleus; TP, posterior tuberculum; V, ventricle; VH, ventral hypothalamic nucleus. Scale bars, 5 μm.

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