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Structure of chicken calcitonin predicted by partial nucleotide sequence of its precursor

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DNA complementary to chicken ultimobranchial gland mRNA was cloned into the Pst I site of plasmid vector pBR322 A plasmid was selected by DNA-mRNA hybridization We report here the partial nucleotide sequence of chicken calcitonin mRNA and the deduced complete amino acid sequence of chicken calcitonin

mRNA Calcitonin Chicken ultimobranchial gland cDNA cloning Nucleotide sequence Amino acid sequence

1. INTRODUCTION

Calcitonin, a 32 amino acid polypeptide produced in mammals by the C cells of the thyroid and in lower vertebrates by the ultimobranchial gland, shows important differences in its amino acid sequence. Based on structure, three groups of calcitonins can be distinguished: (i) human and murine [1,2] (Primate and Rodentia); (ii) bovine, porcine and ovine [3–5] (Artiodactyls); (iii) salmon and eel [6,7] (Teleosteans). They all share in common 9 amino acids in invariant positions. Intragroup differences are minimal: i.e., 2–3 differences in amino acid sequence, while intergroup differences are maximal. Furthermore, the biological potency of non-mammalian calcitonins (eel, salmon) in mammalian species is much higher than that of mammalian calcitonins. No sequence of calcitonin from Sauropsidea (birds and reptiles) has yet been reported. In order to obtain the amino acid sequence of avian calcitonin, we have used genetic engineering to establish the structure of chicken calcitonin, a molecule which has been purified [8], and its primary translation precursor identified by us [9]. We report here the deduced amino acid sequence of chicken calcitonin.

Abbreviations: mRNA, messenger RNA; ss,ds cDNA, single-stranded, double-stranded complementary DNA, bp, base pair

2. MATERIALS AND METHODS

2.1. *Isolation of poly(A^+) rich RNA*

The detailed experimental procedures were described in a previous report [9]. Total RNA was extracted from chicken ultimobranchial glands with phenol-chloroform and purified by LiCl precipitation. Poly (A^+) rich RNA was separated by chromatography on oligo(dT) cellulose (Collab. Res.) and tested by in vitro translation, immunoprecipitation with a purified sheep antiserum against synthetic salmon calcitonin and SDS–polyacrylamide gel electrophoresis (PAGE).

2.2. *Construction of cDNA clones*

The cDNA was constructed as described by Kacian and Myers [10]. First strand synthesis was made by avian myeloblastosis virus reverse transcriptase (a generous gift from J.W. Beard, Life Science Inc.) using poly (A^+) RNA as a template. After alkaline hydrolysis, unincorporated nucleotides were removed by chromatography on Sephadex G-75 (Pharmacia) equili-

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brated with 0.1% SDS–Tris buffer. The second strand was synthesized using ss cDNA as a substrate for reverse transcriptase. The hairpin structure was removed by the action of S1 nuclease.

The ds cDNAs were separated on a 5–20% sucrose gradient and fractions containing full-length species were selected and tailed with poly(dC) using terminal transferase (BRL). The tailed ds cDNAs were inserted into the *Pst*I site of pBR322, after poly(dG) tailing of the linearized plasmid.

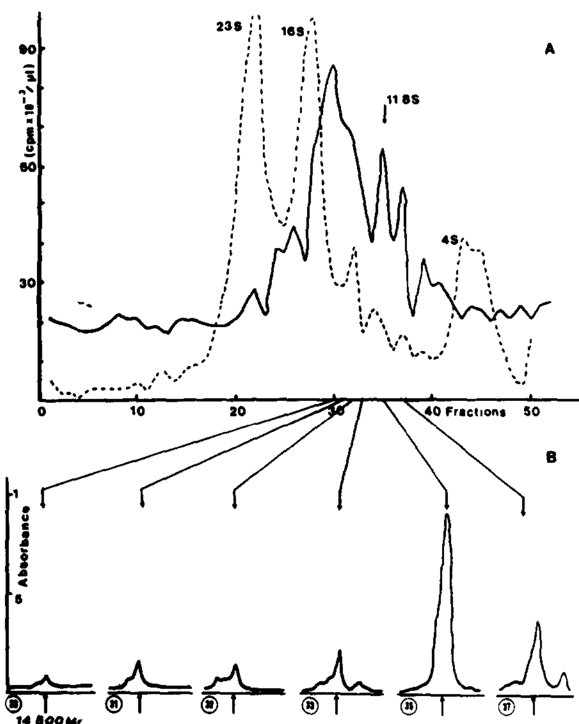

Fig.1 (A) Sucrose gradient fractionation of poly(A^+) RNA from ultimobranchial glands. Poly(A^+) RNA (50 μg) was centrifuged (40000 rpm for 18 h at 4°C) through a 1–30% linear sucrose gradient (---) Radioactive profile of tritium labelled 23 S, 16 S and 4 S ribosomal RNA An aliquot of each fraction was translated in rabbit reticulocyte lysate containing [^{35}S]methionine (2 μCi/μl, 800 Ci/mmol) (——) Radioactivity incorporated into proteins was measured by trichloroacetic acid precipitation Translation products were analysed by SDS–polyacrylamide (15%) gel electrophoresis, after immunoprecipitation with anti-salmon calcitonin serum (B) Densitometric scans (arbitrary units) of autoradiographies of specific immunoprecipitates, corresponding to mRNA fractions directing preprocalcitonin synthesis

Under category II physical containment conditions, *E. coli* strain MM294 was transfected with the hybrid plasmids [11].

2.3. *Clone screening procedure*

Tetracycline resistant and ampicilline sensitive colonies were screened using ^{32}P-labelled cDNA to poly(A^+) RNA extracted from ultimobranchial glands. Two probes were prepared as already described above for ss cDNA: a positive cDNA probe was made against the 12 S fraction, showing maximal translational activity and a negative one with the 4 S fraction (fig.1). Plasmids obtained from colonies giving a positive response with the first probe were further screened by hybrid-selection translation, using the batch technique of Parnes et al. [12].

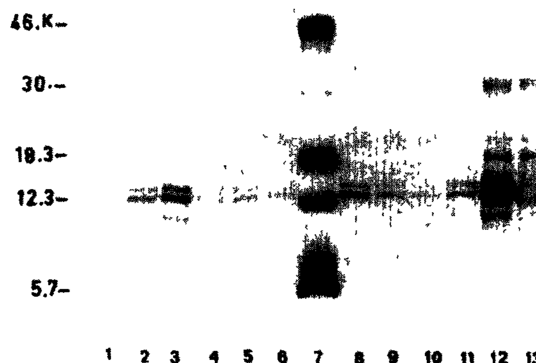

Fig.2. Screening procedure used to identify chicken calcitonin cDNA clones. Poly(A^+) RNA was hybridized (2 h at 50°C) with DNA of plasmids (individual or in batches) immobilized on nitrocellulose papers in 20% formamide. After intensive washing, specific mRNA was then eluted from the DNA-RNA hybrids and precipitated by alcohol. mRNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine. Cell free translation products were immunoprecipitated with anti-salmon calcitonin serum, analysed by SDS–polyacrylamide gel (20%) electrophoresis and autoradiographed. Lanes 1–6. mRNA selected by individual clones; lane 7: ^{14}C-labelled protein M_r standards. insulin, 5766; cytochrome *c*, 12300; lactoglobulin A, 18367, carbonic anhydrase, 30000, ovalbumin, 460000, lanes 8–10· mRNA selected by batches of seven plasmids, lane 11· 1 μg of chicken ultimobranchial poly(A^+) mRNA; lane 12: the same as 11 in the presence of an excess of cold salmon calcitonin.

2.4. *Isolation of plasmid DNA*

DNA of selected plasmids were prepared from bacterial cells, by SDS extraction and purified by centrifugation on a CsCl gradient.

2.5. *DNA sequence analysis*

After mapping of restriction endonuclease cleavage sites, *Sau*3A fragments were isolated and cloned in the *Bam*HI restriction site of single-stranded DNA cloning vectors M13 mp 10 and mp 11 [13]. Both DNA strands were sequenced using the dideoxy method [14].

3. RESULTS

We obtained 800 tetracycline resistant ampicilline sensitive clones, 40 of which showed maximal hybridization with the labelled probe prepared against the 12 S fraction (fig.1a) of ultimobranchial mRNA which had the highest translational activity for preprocalcitonin (fig.1b). Six plasmids selectively retained chicken calcitonin mRNA (fig.2). The partial nucleotide sequence and the deduced amino acid sequence of the insert of plasmid 2947 (fig.2, lane 3) is shown in fig.3.

4. DISCUSSION

Chicken calcitonin, like non-mammalian calcitonins, has a high biological activity and shares with salmon calcitonin a similar amino acid composition [8], and common antigenic sites [9,15]. Elucidation of the nucleotide sequence of both strands of a 141 bp of the insert subcloned in M13 mp 10 and mp 11 revealed that bases 34–129 code for a 32 amino acid peptide showing the characteristic structure of known calcitonins, with the exception of serine instead of asparagine in position 3. As in human [16,17] and murine [18] precalcitonin, chicken calcitonin is preceded by a Lys-Arg cleavage site and followed by a Gly-Lys-Lys-Arg sequence, characteristic of amidation and proteolytic cleavage site. Chicken calcitonin shows a high sequence homology with salmon calcitonin (84%) (fig.4) and with eel calcitonin (94%). Sequence homology with mammalian calcitonins is much lower: 47% in the case of human and murine calcitonins and minimal (31%) with ovine and bovine calcitonins. The amino acid composition of the predicted chicken calcitonin is in complete agreement with the amino acid composition

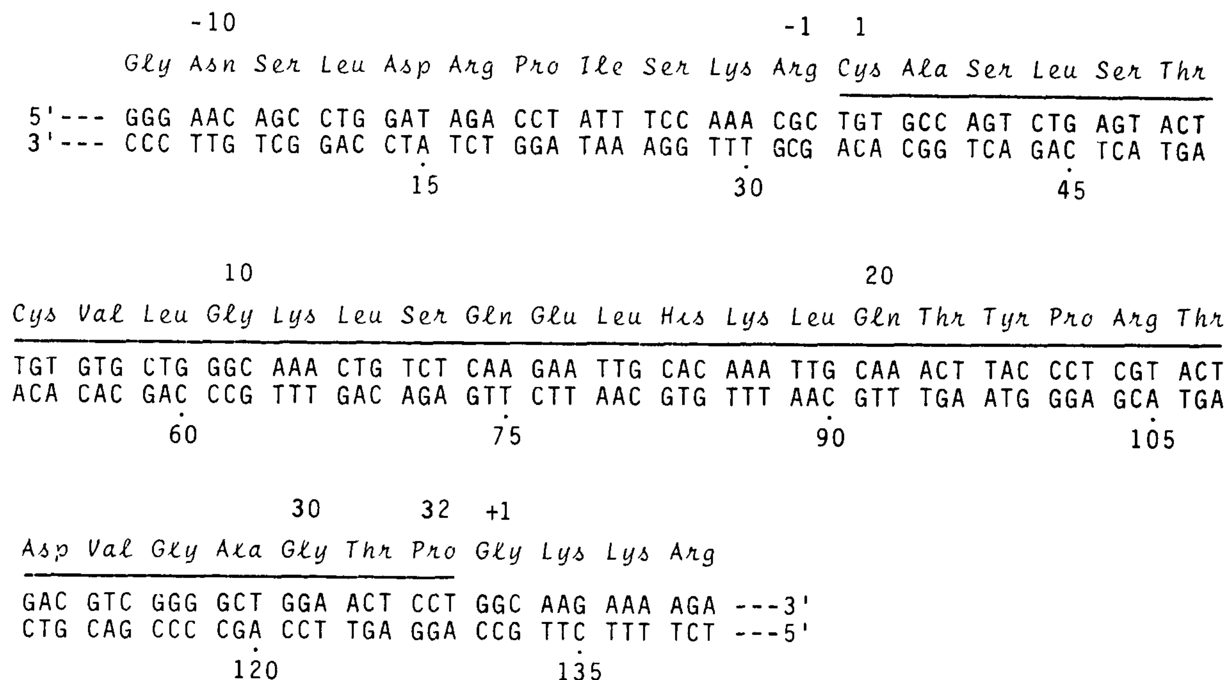

Fig 3. Partial nucleotide sequence of cDNA insert in plasmid 2947 The predicted amino acid sequence encoded by nucleotides 34–129 corresponds to that of chicken calcitonin

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          1       5        10        15        20        25        30
CHICKEN   C A S L S F C V L G K L S Q E L H K L Q T Y P R T D V G A G T P
          *     * * * *   *                                     *       *
EEL       - S N - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
          *   | * * * *   *                                     *       *
SALMON1   - S N - - - - - - - - - - - - - - - - - - - - - - - N T   S - - -
          *   | * * * *   *                                     *       *
SALMON2   - S N - - - - - - - - - - - D - - - - - - F - - - N I - V - A -
          *   | * * * *   *                                     *       *
SALMON3   - S N - - - - M - - - - - - D - - - - - - F - - - N I - V - A -
          *   | * * * *   *                                     *       *
HUMAN     - G N - - - - M - - T Y T - D F N - F H - F - Q - A I - V - A -
          *   | * * * *   *                                     *       *
MURINE    - G N - - - - M - - T Y T - D L N - F H - F - Q - A S - V - A -
          *   | * * * *   *                                     *       *
OVINE     - S N - - - - - - S A Y W K D - N N Y H R Y S G M G F G P E - -
          *   | * * * *   *                                     *       *
BOVINE    - S N - - - - - - S A Y W K D - N N Y H R F S G M G F G P E - -
          *   | * * * *   *                                     *       *
PORCINE   - S N - - - - - - S A T W R N - N N F H R - S G M G F - P E - -
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Fig.4. Comparison of amino acid sequences of chicken, eel, salmon 1,2,3, human, murine, ovine, bovine and porcine calcitonins (—) Amino acids common with chicken calcitonin; (*) amino acids common to all 10 calcitonins, (|) amino acid (Asn) common to all calcitonins except chicken calcitonin

reported by Nieto et al. [8] for extracted and purified chicken calcitonin I. The elucidation of the structure of chicken calcitonin underlines the fact that the large modifications of calcitonin structure have occurred later in evolution and are not associated with the passage from aquatic to terrestrial life.

Chemical synthesis of chicken calcitonin will make available a highly biologically active molecule, which could also be of therapeutic value. Furthermore, generation of the cloned chicken calcitonin probe will permit the isolation of calcitonin gene from a chicken genomic bank, thus establishing the structure of the calcitonin gene in this species.

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