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Cloning and sequence analysis of cDNA for corticotropin-releasing factor precursor from the teleost fish *Catostomus commersoni*

(neuropeptide/hormone precursor/nucleotide sequence/caudal neurosecretory system/hypothalamus)

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ABSTRACT **The sequence of a cDNA encoding the corticotropin-releasing factor precursor has been identified by screening λgt11 libraries constructed from poly(A)$^+$ RNA of the hypothalamic region of the white sucker *Catostomus commersoni* brain with synthetic oligonucleotide probes deduced from the sequence of the rat corticotropin-releasing factor. The amino acid sequence of corticotropin-releasing factor of the sucker is strikingly conserved when compared to its counterpart from rat and differs only in two positions at the carboxyl terminus; in contrast, there is little similarity between their cryptic regions.**

In mammals, secretion of the adrenocorticotropic hormone is stimulated by the 41-amino acid corticotropin-releasing factor (CRF), a neuropeptide hormone (1, 2) that was isolated initially from sheep hypothalamus (3, 4). Meanwhile, structurally related CRF peptides have been found in nonmammalian vertebrates; sauvagine in the skin of the South American frog *Phyllomedusa sauvagei* (5), or urotensin I (UI) in the fish caudal neurosecretory system of the white sucker *Catostomus commersoni* (6), the carp *Cyprinus carpio* (7), and the flathead sole *Hippoglossoides elassodon* (8), suggesting that they too belong to the CRF superfamily. Although the functional role of sauvagine is still unresolved, the UI neuropeptide may be involved in osmoregulation in fish (9). In addition, it has been suggested that UI could function as a fish CRF (10).

The primary structure of the CRF and UI hormone precursors have been analyzed by sequencing, respectively, cDNAs for CRF from the hypothalamus of sheep (11), humans (12), and rats (13) and for UI from the spinal cord of carp (14). As with other neuropeptide precursors, prepro-CRF and prepro-UI consist of a signal peptide, followed by a relatively large cryptic portion of unknown function preceding the CRF or UI peptide; the neuropeptide hormones are separated from the cryptic regions by processing signals, consisting of three or four basic amino acids. The hormones terminate in a typical processing sequence, Gly-Lys, with lysine as proteolytic cleavage site and glycine as CRF amidating signal.

Distinct UI- and CRF-like immunoreactivities have been described in the central nervous system and the pituitary of the sucker using antisera raised, respectively, against sucker UI and rat or ovine CRF (15). These findings are supported by high-pressure liquid chromatography (HPLC) indicating that a CRF-like substance from brain and pituitary extracts of the sucker shows very similar retention times to that for rat CRF but differs markedly from that for the authentic UI peptide (16).

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We report here the identification of a sucker cDNA clone that encodes a previously unknown CRF neuropeptide hormone, which is identical with the human/rat CRF amino acid sequence except for two substitutions.¶||

MATERIALS AND METHODS

Escherichia coli endonuclease-free DNA polymerase I, Klenow enzyme, and restriction enzymes were obtained from Boehringer Mannheim; reverse transcriptase, RNase H, T4 DNA ligase, and T4 DNA polymerase were from P. H. Stehelin & Cie. A.G. (Basel); *E. coli* DNA ligase, *Eco*RI methylase, and *Eco*RI linkers were purchased from New England Biolabs; T4 polynucleotide kinase was from Bethesda Research Laboratories; oligo(dT)$_{12-18}$ and oligo(dT)-cellulose were from Pharmacia; *Eco*RI-digested dephosphorylated λgt11 arms were from Promega Biotec (Madison, WI); Bluescript plasmid and Gigapack Gold *in vitro* packaging extracts were obtained from Stratagene Cloning Systems (La Jolla, CA); and deoxycytidine 5′-[α^{32}P]triphosphate (3000 Ci/mmol; 1 Ci = 37 GBq) and adenosine 5′-[^{32}P]-triphosphate (6000 Ci/mmol) were from Amersham-Buchler (Braunschweig, F.R.G).

Poly(A)$^+$ RNA Preparation and Construction of λgt11 Libraries. Based on immunocytochemical and chromatographic evidence for the presence of a CRF-like neuropeptide in specific areas of sucker brain (15, 16), total RNA was isolated from hypothalamic fragments of 225 fish that had been urophysectomized 8 days prior to dissection to enhance the relative amount of mRNA encoding UI and/or CRF-like molecules (17). The dissected tissue was homogenized and total RNA was selectively precipitated as reported (18). The RNA pellet was dissolved in homogenization buffer (18), layered onto a 5.7 M cesium chloride cushion, and centrifuged at 150,000 × *g* for 20 hr. The resulting RNA pellet was solubilized and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (19).

The cDNA was prepared from 10 μg of poly(A)$^+$ RNA according to the method of Gubler and Hoffmann (20) and inserted into the *Eco*RI site of λgt11 (21). The resulting library consisted of 4.5 × 10^5 independent recombinants as determined prior to amplification. The library was subsequently amplified and phage supernatants were stored at 4°C (21). A second similarly constructed hypothalamic library containing

Abbreviations: CRF, corticotropin-releasing factor; UI, urotensin I.

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¶The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04116).

||Though rat and human CRF possess identical amino acid sequences we have for simplicity referred only to the rat hormone.

1×10^6 independent recombinants was screened without resort to amplification.

Identification of cDNAs Encoding the Sucker CRF Precursor. A total of 9×10^5 amplified recombinants were screened by *in situ* hybridization on nitrocellulose replica filters (≈50,000 phage per 150-mm plate) using the unique 47-mer synthetic oligonucleotide. The probe was 5'-end-labeled with T4 polynucleotide kinase to a specific activity of 4.46×10^7 cpm/μg. The filters were hybridized as reported (22) except that the wash temperature was reduced to 30°C. Positive plaques from this initial screen were then rescreened with the 17-mer synthetic oligonucleotide pool. The probe mixture was 5'-end-labeled as indicated above to a specific activity of 2.45×10^7 cpm/μg and filters were hybridized as described (14). Screening of the second hypothalamic library was carried out using a 0.7-kilobase-pair (kbp) CRF-encoding fragment isolated from one of the primary clones and labeled to a specific activity of 2×10^8 cpm/μg (23). Filters were hybridized and washed by standard procedures (24).

Nucleotide Sequence Analysis. Inserts from positive clones were subcloned into Bluescript plasmids or into M13mp8 (25) for sequence analysis (26). Clones corresponding to the sucker CRF precursor were unambiguously identified by alignment of the nucleotide sequence with that for rat CRF using the ALIGN program of the DNASTAR sequence analysis software package (Madison, WI), which is based on the Wilbur and Lipman logarithm (27).

RESULTS

Screening Strategy. The search for a sucker CRF was based on recent immunocytochemical and HPLC analysis suggesting that a rat CRF-like peptide is present in the brain of teleosts (15, 16). The very similar retention times of the sucker peptide and rat CRF on HPLC columns point to structurally related hormones being different in only a few amino acid residues. Hence a unique 47-mer synthetic oligonucleotide corresponding to the most frequently used codons in fish (28) was deduced from the amino acid sequence of rat CRF_{17-32} (Fig. 1), which shows considerable similarities with other known mammalian CRFs (11, 12) but significantly less with the teleost neuropeptide UI (14). This probe was used to screen a sucker brain hypothalamic region λgt11 library. Clones giving positive hybridizing signals were rescreened with a 17-mer synthetic oligonucleotide pool representing all possible sequences deduced from rat CRF_{17-22} (Fig. 1).

Analysis of 9×10^5 recombinants using the 47-mer probe gave rise to a total of six positively hybridizing clones falling into two families as judged by hybridization signal intensity and displaying inserts between 0.7 and 2.0 kbp. Three of these clones also gave signals with the 17-mer oligonucleotide pool. Nucleotide sequence analysis showed that these three clones represented identical CRF clones with 0.7-kbp inserts. The unique synthetic 47-mer probe was found to contain two long stretches of uninterrupted homology with the cloned sucker CRF-encoding cDNA, 17 and 10 residues long, respectively (Fig. 1). The overall homology of the 47-mer probe with the sucker CRF cDNA was 82%, while it showed only 51% sequence identity with the carp UI cDNA (14).

Since, even after rescreening with the 0.7-kbp fragment, the first library did not yield a clone encoding the complete sucker CRF precursor, a second hypothalamic library of 1×10^6 recombinants was screened using the 0.7-kbp fragment encoding CRF. This yielded 58 hybridization positives of which five of eight clones analyzed contained inserts of ≈1.0 kbp; these were subsequently shown to encode the complete fish CRF precursor.

Sequence of the Sucker CRF Precursor. Fig. 2*A* shows the sequence and Fig. 2*B* the restriction map of the 1.0-kbp fragment. The 1.0-kbp cloned cDNA fragment exhibits a single open reading frame predicting a protein of 162 amino acid residues, including a CRF neuropeptide of 41 amino acid residues at the carboxyl terminus. As expected from the HPLC data (16) sucker and rat CRF differ in two amino acids only; in positions CRF_{37} and CRF_{41}, rat leucine is replaced by methionine and rat isoleucine by phenylalanine, respectively. As is the case for mammalian CRF precursors (11–13), sucker CRF is framed by potential processing and cleavage signals, with a tetrapeptide Arg-Xaa-Arg-Arg at the amino terminus and Gly-Lys at the carboxyl terminus of the precursor sequence. The presence of a proximal glycine residue as a potential amide group donor suggests that the mature sucker CRF is amidated. The translation initiation site is provisionally assigned to the methionine codon shown at position 106 of the nucleotide sequence. The first 24 amino acids exhibit features characteristic for signal peptides of secretory proteins (29), including a high proportion of hydrophobic amino acids. The signal sequence probably terminates at either residue 24 (alanine) or 27–29 (serine) (Fig. 2*A*).

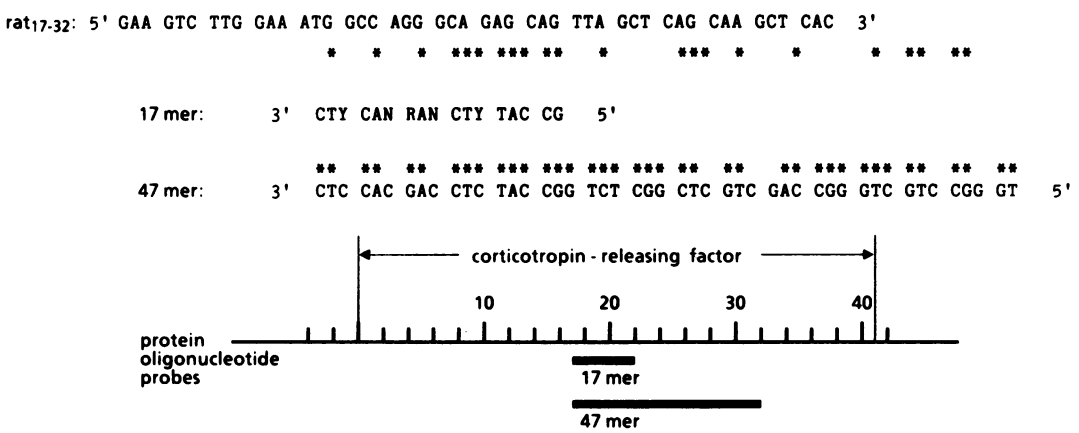

FIG. 1. Synthetic oligonucleotides deduced from rat CRF amino acid sequence. Rat CRF_{17-32} and the corresponding mRNA sequence is shown on the top line; identical nucleotides of rat CRF and carp UI (14) are indicated by asterisks underneath the rodent nucleotide sequence; the sequence identity in this region is ≈47%. The 17-mer represents all possible cDNA sequences corresponding to rat CRF_{17-22}. The unique 47-mer oligonucleotide has been deduced from rat CRF_{17-32}. Degenerate code combinations are given according to the rules of the International Union of Biochemistry: Y, C or T; N, A, G, C, or T; R, A or G. Oligonucleotides were synthesized in an automated DNA synthesizer by the phosphoramidite method. Asterisks above the 47-mer oligonucleotide indicate positions of homology between the synthetic probe and the corresponding region of the isolated sucker CRF-encoding cDNA.

A

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rat 5' AAACTCAGAGCCCAAGTACGTTGAGAAACTGAAGAGAAAGGGGAAAGGCAAAGAAAAGGA   60

GAAGAGAAAGGAGAAGAGGAAGAAAACCTGCAGGAGGCATCCTGAGAGAGGTACCTCGCAGAACAACAGT  130

sucker 5'          TCGGCACGAGCAACTGCAAGAGAGAGACGCATCTCGCAAAGTTCCAAAACCATCCAA   57
rat    GCGGGCTCACCTGCCAAGGGAGGAGAAGAGAGCGCCCCTAAACATGCGGCTGCGGCTGCTGGTGTCCGCG  200
                                                   m  r  l  r  l  l  v  s  a

                                                m  k  l  n  f  l  v  t
GGATATACCAATTACGCACACCACAGATTCTTCTCGCCACTCTTTGACATGAAGCTCAATTTTCTCGTCA  127
GGCATGCTGCTGGTGGCTCTGTCGCCCTGTCTGCCTTGCAGGGCCCTGCTGAGCAGGGGATCCGTCTCTG  270
g  m  l  l  v  a  l  s  p  c  l  p  c  r  a  l  l  s  r  g  s  v  s  g

  t  v  a  l  l  v  a  f  p  p  p  y  e  c  r  a  i  d  s  s  s  n  q
CCACCGTGGCTCTACTCGTTGCCTTTCCACCACCTTATGAATGTAGAGCCATAGACAGCAGCTCAAACCA  197
GAGCGCCGCGGGCCCCGCAGCCGTTGAATTTCTTGCAACCGGAGCAGCCCCAGCAACCTCAGCCGATTCT  340
  a  p  r  a  p  q  p  l  n  f  l  q  p  e  q  p  q  q  p  q  p  i  l

  p  a  t  d  p  d  g  e  r  q  s  a  p  v  l  a  r  l  g  e  e  y  f
GCCAGCCACGGACCCCGATGGAGAGCGACAGTCGGCACCGGTTCTGGCGCGCTTGGGAGAGGAGTACTTC  267
GATCCGCATGGGTGAAGAATACTTCCTCCGCCTGGGGAACCTCAACAGAAGTCCCGCTGCTCGGCTGTCC  410
  i  r  m  g  e  e  y  f  l  r  l  g  n  l  n  r  s  p  a  a  r  l  s

i  r  l  g  n  r  y  q  n  s  l  r  s  s  p  d  t  y  p  e  t  s  q  y
ATCCGGCTCGGTAACAGATATCAGAATTCTCTCCGGTCTTCACCTGACACGTACCCCGAGACATCCCAGT  337
CCCAACTCCACGCCCCTCACCGCGGGTCGCGGCAGCCGCCCCTCGCACGACCAGGCTGCGGCTAACTTTT  480
p  n  s  t  p  l  t  a  g  r  g  s  r  p  s  h  d  q  a  a  a  n  f  f

  p  k  r  a  l  q  l  q  l  t  q  r  l  l  e  g  k  v  g  n  v  g  r
ATCCCAAAAGAGCACTGCAGCTCCAGTTAACGCAGCGTCTGTTGGAGGGCAAAGTTGGAAACGTCGGCCG  407
TCCGCGTGTTGCTGCAGCAGCTGCAGATGCCTCAGCGCCCGCTCGACAGCAGCACGGAGCTGGCGGAACG  550
  r  v  l  l  q  q  l  q  m  p  q  r  p  l  d  s  s  t  e  l  a  e  r
                                                    ⇓ C R F
  w  d  g  n  y  a  l  r  a  l  d  s  e  e  r  e  r  r  s  e  e  p  p
ATGGGATGGCAATTACGCGCTCCGGGCGCTCGACTCCGAGGAGAGAGAGCGCAGGTCGGAGGAGCCGCCG  477
CGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAGAGGGAGAGGCGGTCCGAGGAGCCGCCC  620
  g  a  e  d  a  l  g  g  h  q  g  a  l  e  r  e  r  r  s  e  e  p  p
                                                    ⇑ C R F
  i  s  l  d  l  t  f  h  l  l  r  e  v  l  e  m  a  r  a  e  q  l  a  q
ATTTCCCTGGATCTGACCTTTCATCTGCTACGAGAAGTACTAGAGATGGCCAGAGCCGAACAATTGGCCC  547
ATCTCTCTGGATCTCACCTTCCACCTTCTGAGGGAAGTCTTGGAAATGGCCAGGGCAGAGCAGTTAGCTC  690
  i  s  l  d  l  t  f  h  l  l  r  e  v  l  e  m  a  r  a  e  q  l  a  q
                                                    ⇓
  q  a  h  s  n  r  k  m  m  e  i  f  g  k  ter
AGCAAGCTCACAGCAACCGCAAAATGATGGAAATATTCGGGAAGTAACCGCGACCAAACCCATCTGCCAA  617
AGCAAGCTCACAGCAACAGGAAACTGATGGAGATTATCGGGAAATGAAATGTTGCGCTTGGCCAAAACGA  760
  q  a  h  s  n  r  k  l  m  e  i  i  g  k  ter
                                                    ⇑
AGATATTTTTACATTTAGCACAAACATAAATATTCTGTACCATAGTGCTGCTTTTCCATCACGATCTATT  687
TTCTGCATTTAGCACACAAGTAAAAATAAAAAATTTAAAACACAGTATTCTGTACCATACTGCAGCTCTG  830

TATACCGGTGAATTACAGTATGTATTTGTAGATATTTCAATGAAGGCGAATAGAGTTGGTGTATATGACC  757
ATATCATTTGTTTATTTTTATATAGCTTGAAGCATAGAAGATGTACAGGGAGAGAGCCTATATACCCCTT  900

ATGACTGGTAAATTGTTCAGATGCGCAAAACTGTCATCAGTCTCCATCAGTGTTATTGTATAAGTGATCA  827
AATTAGCATGCACAAAGTGTGTTTCTTTGTAGTAACAAAACAGCGTTATTTGTATTGTTCACGCTTAGTT  970

TTCAGAGCTGTAGATGATTCAAAACGGATTTCTTAAAAAAGAAAGAAGAATTTAATATTTTCAAGTACTT  897
TCTATGTGCAAATAAGTGTCTTTATAGCGATATCTTAAAGAAAATGTGGATCCAAGGAGGAAACCTTTAA 1040

TATTATTTTATCTGACAGACTTGAGTTTCATTGTAACATACAGTTACATTGTTTAAGTTTGTTAATAAAC  967
AAAAGCAGATGGAAGTCACCCAGTTGTTTTTATTTGGAGACACAGTGTAAGAGAATTCATTCTTGAGGGG 1110

TAATGAGCAACCATTG 3'                                                      983
TGGCTAGGACAAAATGTGTAAGCTCTTTGAATCAACTTTTTCTTGTAAATGTTTCAATAATAAAACATCT 1180

TTCTGATCCTTGGTC 3'
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B

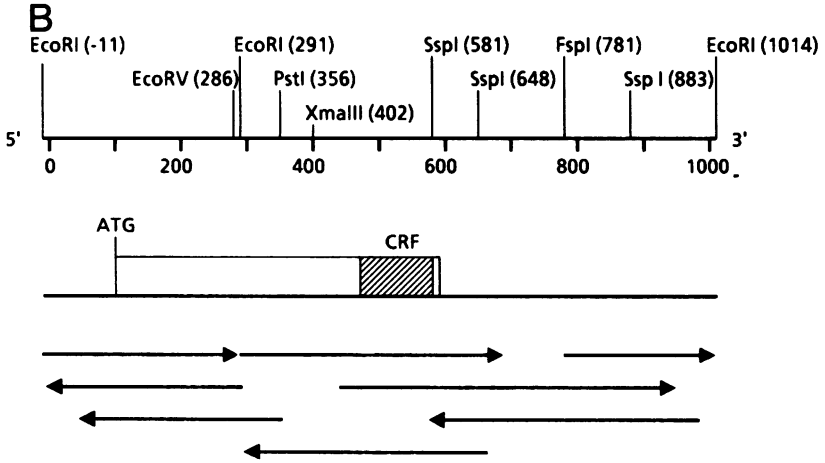

FIG. 2. (*A*) Nucleotide sequence and deduced amino acid sequence of sucker CRF precursor cDNA compared with the counterpart from rat. Nucleotide residues are numbered on the right. CRF peptides are framed by arrows. The underlined sequences represent conserved motifs found in all 3′ noncoding CRF sequences. The overline in the sucker sequence (positions 961–966) indicates the position of the polyadenylylation signal. A conserved nucleotide region was noted at positions 247–285 (fish) and 345–383 (rat) encoding 13 amino acid residues of which 10 are identical and a further 1 is a conservative change. The first and the last amino acids represent potential processing signals. The single letter convention for amino acids is used. a, Ala; c, Cys; d, Asp; e, Glu; f, Phe; g, Gly; h, His; i, Ile; k, Lys; l, Leu; m, Met; n, Asn; p, Pro; q, Gln; r, Arg; s, Ser; t, Thr; v, Val; w, Trp; y, Tyr; z, Glx. (*B*) Restriction map. The sequence corresponding to the coding region is indicated by the box. The *Eco*RI sites at each end of the clone are contributed by the *Eco*RI linkers. The whole insert or fragments thereof were subcloned into the *Eco*RI site of Bluescript KS M13(+) plasmid or M13-mp8 for sequence analysis by the dideoxy method (26). The length and direction of sequencing are shown by arrows.

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                    1        10        20        30        40
Sucker CRF      RERR:SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKMMEIF:GK
Rat CRF (12)    RERR:SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII:GK
Human CRF (11)  RERR:SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII:GK
Sheep CRF (10)  RKRR:SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA:GK
Sucker UI (7)       NNDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV
Sole UI (8)         SEEPPMSIDLTFHMLRNMIHRAKMEGEREQALINRNLLDEV
Sauvagine (5)        EGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI
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FIG. 3. Comparison of amino acid sequences of sucker CRF and the other known CRF-related peptides (numbered sequentially from amino terminus). Sequences have been taken from the references indicated in parentheses. Where known, the flanking amino acids are indicated separated from the hormone sequence by two dots. Note that the porcine CRF differs from rat/human by only two residues, whereas the bovine and caprine CRFs are more closely related to the ovine peptide (30, 31), and that the rat CRF differs from sucker CRF by only two residues (7). The single-letter amino acid code is used.

Alignment of the amino acid sequence of sucker and rat (13) CRF precursors reveals considerable relationship between the respective hormone regions including the processing signals at the amino and carboxyl termini, but a lesser similarity between the cryptic portion of the fish and rat precursors. Comparison of the nucleotide sequence of various CRF-encoding cDNAs demonstrates a high degree of conservation between the mammalian species (11–13). In contrast, the correspondence is somewhat lower between mammalian and sucker CRF cDNA. Attempts to determine the size of poly(A)$^+$ RNA encoding prepro-CRF by Northern blot analysis of mRNA from sucker brain were unsuccessful, suggesting that sucker CRF mRNA, like its mammalian (11) counterpart, is expressed at very low abundance.

DISCUSSION

The data presented here show that a mammalian CRF counterpart is expressed in the hypothalamic neurosecretory system of the sucker *C. commersoni*, suggesting that at least in this fish species two members of the CRF superfamily are produced, of which the neuropeptide UI is present primarily in the urophysis, possibly also in the tuberal region of the hypothalamus (10, 15, 16). CRF may be present in the region encompassing both the nucleus preopticus and nucleus lateralis tuberis. UI is known to have potent vasodilatory hypotensive effects in mammals as well as corticotropin-releasing activity in mammals and fish (6, 10) and may therefore play a major role in osmoregulation in fish (9). It remains to be determined whether the activity spectra of the sucker CRF resemble those of the mammalian CRFs or of UI, or of both.

As expected from previous immunocytochemical studies (10, 15–17), the chemical structure of sucker CRF is closer to rat/human (12, 13) and porcine CRF (30) than to ovine (11), caprine, or bovine CRF (31, 32) (Fig. 3). The primary structure of the sucker CRF precursor indicates a similar organization to its mammalian counterparts, suggesting that CRF and its related peptides (UI and sauvagine) evolved from a common ancestor by gene duplication. The high conservation of the hormone sequence compared with the low similarities between the more heterogeneous cryptic portion of the precursors may imply that the latter moiety serves primarily a structural function. This is unlike the vasopressin/oxytocin superfamily where, in addition to hormones, their neurophysin carriers are also highly conserved throughout the animal kingdom (10). The earlier observations that part of the cryptic portion of ovine pro-CRF is related to amino-terminal sequences of the vasopressin–neurophysin precursor (11) cannot be confirmed for the sucker sequence.

At the nucleotide sequence level, the similarities between sucker and rat CRF-encoding regions are almost 80%. Apart from this there is a lesser homology between the rest of the sucker and rat sequence with the exception of two conserved motifs immediately 3′ to the stop codon, which are present in all known CRF sequences (Fig. 2*A*, underlined).

A CRF peptide based on the predicted amino acid sequence was synthesized (J. Rivier, Y.O., and K.L., unpublished observations). Preliminary observations suggest a potency similar to that of rat/human CRF or of UI in stimulation of goldfish pituitary corticotropin secretion (T. Tran, J. Fryer, Y.O., and K.L., unpublished observations).

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