Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5485–5489, September 1983
Biochemistry

Mammalian pancreatic preproglucagon contains three glucagon-related peptides

(glucagon precursor/oligodeoxynucleotide/prohormone processing)

LINDA C. LOPEZ*†, MARSHA L. FRAZIER*, CHUNG-JEY SU*, ASHOK KUMAR‡, AND GRADY F. SAUNDERS*†

*Department of Biochemistry, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030; †University of Texas Graduate School of Biomedical Sciences, Houston, Texas 77030; and ‡Department of Anatomy, University of Texas Health Science Center, San Antonio, Texas 78284

Communicated by Norman Hackerman, May 26, 1983

ABSTRACT **We have isolated cDNA clones encoding bovine pancreatic preproglucagon. Twenty-five putative preproglucagon clones were selected by screening 3,100 clones of a fetal bovine pancreas cDNA library with a synthetic oligodeoxynucleotide probe. The probe was a mixture of synthetic 17-base DNA oligomers constructed to correspond to the six carboxyl-terminal amino acids (residues 24–29) of mature glucagon. Restriction mapping of six of these clones suggested that they represented a single mRNA species. Primary sequence analysis of one clone containing a 1,200-base-pair DNA insert revealed that it contained an essentially full-length copy of glucagon mRNA. Analysis of the cDNA suggested a protein coding sequence of 540 nucleotides and 5′- and 3′-untranslated regions of 90 and 471 nucleotides, respectively. This cDNA sequence encoded a 20-amino acid signal sequence followed by one for glicentin, a 69-amino acid polypeptide containing an internal glucagon moiety that has been found in porcine intestines. Glicentin is followed by two additional glucagon-like peptides, each flanked by paired basic amino acids (Lys, Arg) characteristic of prohormone processing. These polypeptide sequences show striking homology with those for glucagon and other members of the glucagon family of peptides.**

Glucagon is a 29-amino acid polypeptide hormone produced in the α cells of the pancreatic islets (1). As with other polypeptide hormones, the synthesis of glucagon is thought to involve a larger precursor, which is then enzymatically cleaved to the functional form (2). Molecules of various sizes, all larger than glucagon and displaying glucagon-like immunoreactivity, have been isolated from the pancreatic tissues of many different species (3–10). Additional high-molecular-weight forms have been found in gastric and intestinal mucosa (11, 12) and in plasma (13). Glicentin, a putative proglucagon fragment, is a 69-amino acid polypeptide isolated from porcine intestine that has been shown to contain the glucagon sequence in residues 33–61 (14); sequence 1–30 represents the glicentin-related pancreatic peptide (GRPP) isolated from porcine pancreas (15). Glucagon is a member of a family of structurally related peptides that includes secretin (16), glicentin (14), gastric inhibitory peptide (17), vasoactive intestinal peptide (18), prealbumin (19), peptide HI-27 (20), and a growth hormone-releasing factor (21).

In mammals, the primary physiological effect of glucagon is in the liver, where it affects glucose metabolism by inhibiting glycogen synthesis, stimulating glycogenolysis, and enhancing gluconeogenesis (22, 23). *In vitro* studies have shown that glucagon increases the mobilization of glucose, free fatty acids, and ketone bodies, which are metabolites produced in excess in diabetes mellitus (24). For these reasons, interest has focused on the biosynthesis of glucagon and related peptides.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Residue Number	24	25	26	27	28	29
Amino Acid	gln	trp	leu	met	asn	thr
mRNA Sequence	5′- CAA	UGG	UUA	AUG	AAU	AC_ -3′
	G		G		C	
			CUA			
			G			
			C			
			U			
17-mer	3′- GTT	ACC	AAA	TAC	TTA	TG_ -5′
	C		G C		G	
			C			
			T			

FIG. 1. Sequence of the glucagon oligodeoxynucleotide probe (17-mer). Sequences of the mRNA and cDNA corresponding to glucagon amino acid residues 24–29 are shown.

Studies of anglerfish glucagon have elucidated some steps involved in its biosynthesis. At least two distinct mRNA species are found in anglerfish islets, encoding glucagon-related proteins of 14.5 kilodaltons (kDa) and 12.5 kDa (25–27). The mRNA coding for the 14.5-kDa molecule contains sequences that when translated could give rise to glucagon and a second glucagon-like peptide by simple enzymatic cleavage (28). Pulse-chase experiments on isolated rat islets have identified an 18-kDa glucagon-related protein that exhibits prohormone-like kinetics of formation and processing (9). Allowing for a signal peptide, this suggests a mammalian preproglucagon of ≈21 kDa.

We fractionated fetal bovine pancreas poly(A)$^+$ RNA according to size and identified an enriched glucagon-coding fraction by hybridization with a specific oligodeoxynucleotide probe. We used these RNAs as templates to prepare cDNAs and cloned their corresponding recombinant plasmids in bacteria. In this paper, we report the isolation and characterization of clones encoding mammalian preproglucagon. The coding sequence of 540 nucleotides predicts an initial precursor polypeptide of 21 kDa. The nucleotide sequence of this cloned cDNA reveals that the glucagon moiety is contained within a glicentin-coding sequence, which is followed in turn by sequences for two additional glucagon-related peptides.

MATERIALS AND METHODS

Oligodeoxynucleotide Probes. Two oligodeoxynucleotide mixtures were synthesized to correspond to the regions of glucagon coding for amino acids 24–29 (Gln-Trp-Leu-Met-Asn-Thr, a 17-base oligomer) (Fig. 1) or amino acids 24–28 (a 14-base

Abbreviations: kDa, kilodalton(s); bp, base pair(s); GRPP, glicentin-related pancreatic peptide; GLP, glucagon-like peptide.

oligomer, kindly provided by Marvin Caruthers, Dept. of Chemistry, University of Colorado, Boulder, CO). The oligodeoxynucleotides were synthesized by using solid-phase aminophosphite triester chemistry (29, 30).

Construction of the cDNA Library and Identification of Clones Containing cDNAs Coding for Preproglucagon. Total RNA was extracted from second-trimester fetal bovine pancreas as described (31, 32). Poly(A)$^+$ RNA isolated from total RNA by two passes over oligo(dT)-cellulose as described (33) was fractionated by centrifugation on 15–30% linear sucrose gradients. Poly(A)$^+$ RNA from each fraction was dot-blotted onto nitrocellulose by the method of Thomas (34) and hybridized as described (35) with the 14-base oligomer (14-mer), which had been phosphorylated (36) with [γ-^{32}P]ATP (3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq; Amersham) and T4 polynucleotide kinase (Boehringer Mannheim). Poly(A)$^+$ RNA from each fraction was translated in a cell-free system (37) and the products were analyzed by electrophoresis on 15% NaDodSO$_4$/polyacrylamide gels (38). A RNA fraction of ≈14–15 S hybridized specifically with the oligodeoxynucleotide probe, and its translation products were found to be enriched for polypeptides in the range of ≈21 kDa, the expected size of preproglucagon (data not shown). This poly(A)$^+$ RNA was used in the preparation of double-stranded cDNA, which was inserted into the *Pst* I site of bacterial plasmid pBR322 with dG:dC homopolymeric tailing as described by Land *et al.* (39). The recombinant plasmids were used to transform *Escherichia coli* strain RRI (40).

Bacterial colonies containing recombinant plasmids were screened by the colony hybridization assay of Grunstein and Hogness (41). Utilizing a double-positive identification scheme, one set of filters was hybridized with ^{32}P-labeled 17-mer as described (35), and a duplicate set of filters was hybridized with a ^{32}P-labeled cDNA probe. The latter probe was prepared by using the 17-mer as a primer for the synthesis of cDNA (42) with the enriched poly(A)$^+$ RNA fraction used to prepare the library as template. The rationale for utilizing the double-positive hybridization scheme was that colonies containing glucagon-coding sequences should hybridize with both probes. Therefore, colonies showing hybridization with only one of the probes were unlikely to contain glucagon-coding sequences and, in this context, were considered to be false-positives.

For primary sequence analysis of the cloned preproglucagon cDNA, selected restriction fragments were subcloned into the bacteriophage vector M13. DNA was subjected to sequence analysis by the Sanger dideoxy chain-termination method as described (43, 44).

Identification of Glucagon-Coding Poly(A)$^+$ RNA. Poly(A)$^+$ RNA was fractionated on agarose gels in the presence of 10 mM CH_3HgOH and transferred to sheets of nitrocellulose as described (34, 45). The glucagon cDNA recombinant plasmid was ^{32}P-labeled by nick-translation (46) and hybridized with the RNA fixed to nitrocellulose as described (34).

RESULTS

Fractionation of fetal bovine pancreas poly(A)$^+$ RNA on sucrose gradients resulted in an ≈10-fold enrichment for sequences hybridizing with the ^{32}P-labeled 14-nucleotide glucagon-specific probe. Initial experiments indicated that under the hybridization conditions necessary for screening the cDNA library, hybridization with the bacterial host DNA occurred (data not shown). Therefore, a 17-mer was synthesized and used in subsequent experiments. Colony screening with the ^{32}P-labeled 17-mer showed specific hybridization with plasmid DNA in a number of colonies. Screening a duplicate set of colony filters with the ^{32}P-labeled cDNA synthesized by using the 17-mer as primer produced 26 colonies exhibiting hybridization with both probes. Southern blot analysis (47) (with ^{32}P-labeled 17-mer as the hybridization probe) of plasmid DNA from each of the 26 colonies indicated that only one of the selected clones was a false-positive and that all other recombinant plasmids contained cDNA inserts of 850–1,200 base pairs (bp).

Six of the recombinant plasmids were selected at random and grown in quantity for restriction mapping analyses to determine whether one or several mRNA species were represented. Restriction endonuclease digestions and Southern blot analyses of these clones suggested that they represented a single mRNA species. At least one clone, bg13A7 (with an insert of ≈1,200 bp) was deemed likely to represent essentially the entire sequence of the mRNA for pancreatic preproglucagon.

A detailed restriction map of clone bg13A7 was constructed to determine restriction fragments suitable for subcloning into the M13 bacteriophage vector for primary sequence analysis. In most regions, both the sense and nonsense strands were subjected to sequence analysis (Fig. 2). All areas were subjected to sequence analysis at least twice, and all regions overlapping junctions between fragments were sequenced. A second clone, bg4E11, whose inserted DNA is in the orientation opposite to that of bg13A7, was utilized in confirming the 5′-terminal sequence.

Analysis of the primary sequence of the cloned DNA (Fig. 3) suggests that it encodes a 21-kDa initial translation product of 180 amino acids. The coding sequence of 540 nucleotides is preceded by a 5′-untranslated region of 90 nucleotides and followed by a longer 3′-untranslated region of 471 nucleotides, resulting in a total of 1,101 nucleotides. The remainder of the inserted DNA is the dG- and dC-homopolymeric extensions and a poly(A) tract at the 3′ terminus. The first initiation codon, ATG (methionine), in the longest open reading frame of the

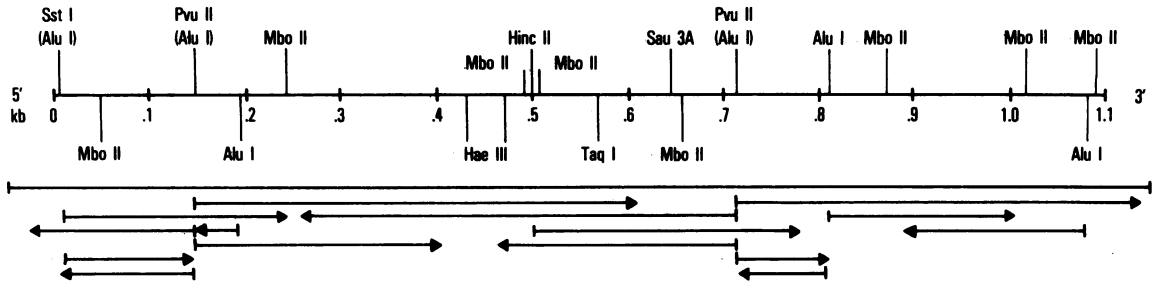

FIG. 2. Partial restriction endonuclease map and strategy for sequence analysis of cloned preproglucagon cDNA. Numbers below the restriction map refer to the positions within the cDNA in kilobases. The direction and extent of sequence determinations are shown by horizontal arrows. Fragments subjected to sequence analysis were from pbg13A7 (with a 1,200-bp cDNA insert), with the exception of the fragment extending from the first *Pvu* II site to the 5′ terminus, which came from pbg4E11. Not included are the dC- and dG-homopolymeric extensions and the poly(A) tract.

nucleotide sequence is located 90 nucleotides from the 5′ end of the cDNA. The initial methionine is followed by 19 predominantly hydrophobic amino acids, which is characteristic of the signal peptide sequences found in secretory proteins (48). The first arginine following the putative signal peptide is likely to be the initial amino acid residue of proglucagon. Amino acids 1–69 (designated by arrows) represent a sequence that shares 94% homology with the putative proglucagon fragment glicentin. Glucagon is contained within this sequence at residues 33–61, flanked by paired basic amino acid residues (Lys, Arg) characteristic of prohormone processing (2). Following the octapeptide extension at the carboxyl terminus of glucagon are two additional glucagon-like peptides (GLP-I and GLP-II) of 37 and 33 amino acids, respectively. Like glucagon, these peptides are flanked by paired basic residues and are separated from each other by a 13-amino acid peptide sequence. The coding sequence for GLP-II is followed by an ochre stop codon, 471 nucleotides of 3′-untranslated sequence, and the poly(A) tract characteristic of most eukaryotic mRNAs (49). Twenty nucleotides upstream from the poly(A) tract is the sequence A-A-T-A-A-A, thought to represent a polyadenylylation signal (50). Although a second possible polyadenylylation site is located ≈120 nucleotides downstream from the stop codon, there is no evidence that this site is utilized.

When the ^{32}P-labeled bg13A7 DNA insert was hybridized with RNA transfer blots of fetal bovine pancreas poly(A)$^+$ RNA, a single RNA species of ≈1,250 nucleotides was detected (Fig. 4). This result indicates that the glucagon clone represents an essentially full-length copy of the mRNA coding for pancreatic preproglucagon.

```
5'...AACAGAGCTCAGGACACTGCACACCCAAACGAGGGCTCACTCTCTCTTCACCTGCTCTGTTC    62
                                    -20
                                   [met] lys ser leu tyr phe val ala gly
CACCTCCTGGTGTCAGAAGGCAGCAAAA        ATG AAA AGC CTT TAC TTT GTG GCT GGA   117
    -10                                     -1  ↓ Glicentin
leu phe val met leu val gln gly ser trp gln arg ser leu gln asn thr
TTG TTT GTA ATG CTG GTA CAA GGC AGC TGG CAA CGT TCC CTT CAG AAC ACA   168
           10                                       20
glu glu lys ser ser ser phe pro ala pro gln thr asp pro leu gly asp
GAG GAG AAA TCC AGT TCA TTC CCA GCT CCC CAG ACC GAC CCG CTC GGC GAT   219
                    30          Glucagon                    40
pro asp gln ile asn glu asp [lys arg] his ser gln gly thr phe thr ser
CCA GAT CAG ATC AAT GAA GAT AAG CGC CAC TCG CAG GGC ACA TTC ACC AGT   270
                                50
asp tyr ser lys tyr leu asp ser arg arg ala gln asp phe val gln trp
GAC TAC AGC AAG TAC CTG GAC TCC AGG CGT GCC CAG GAC TTC GTG CAG TGG   321
      60                                  ↓ 70       Glucagon-
leu met asn thr [lys arg] asn lys asn asn ile ala [lys arg] his asp glu
TTG ATG AAT ACC AAG AGA AAC AAG AAT AAC ATT GCC AAA CGT CAT GAT GAA   372
like peptide I      80                              90
phe glu arg his ala glu gly thr phe thr ser asp val ser ser tyr leu
TTT GAG AGA CAT GCT GAA GGG ACC TTT ACC AGT GAT GTA AGT TCT TAT TTG   423
                                100
glu gly gln ala ala lys glu phe ile ala trp leu val lys gly arg gly
GAA GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA GGA   474
    110                                 120
[arg arg] asp phe pro glu glu val asn ile val glu glu leu arg [arg arg]
AGG CGA GAT TTC CCA GAA GAA GTC AAC ATC GTT GAA GAA CTC CGC CGC AGA   525
Glucagon-like peptide II                            140
his ala asp gly ser phe ser asp glu met asn tyr val leu asp ser leu
CAC GCC GAT GGC TCT TTC TCT GAT GAG ATG AAC ACT GTT CTC GAT AGT CTT   576
                          150
ala thr arg asp phe ile asn trp leu leu gln thr lys ile thr asp [arg]
GCC ACC CGA GAC TTT ATA AAC TGG TTG CTT CAG ACG AAA ATT ACT GAC AGG   627
160
[lys] OC
AAG TAA  GTGTGTCATTCATTACTCAAGATCATCTTCACAATATCACCTGCCAGCCATGTGGGAT   691

GTTTGAAATTTTAAGTTCTGTAAATTTAACAGCTGTATTCTAAAGCCATATTGCTTGCATGCAAATA   758

AATAAATTTCCTTTTAATATTGTATAACCAAAAGATTATAAATTGAATACACCATTGTCAAAATAGT   825

GCTAAAATATCAGCTTTAAAATATGTTAATTCAGAATTCTCTTTCTTTTCTTCTGCTAACCTGCTTA   892

GCAATGAAATTATTTCTCTGTGATATAATTTGTATATATAAATTACTCCAATCACAACATATTTGCA   959

TTATAATAAGATAAGGGGAAGGACTGGTAGCCACAGTTGTGAAATGGGAAAGAGAATTTTCTTCTTG  1026

AAACTTTTGTCATAAAAATGCTCAACTTTCAGTATATAAAAGATAAACTAAATAAAAATTTCAAGCT  1093

TCTTCATCAAAAAAA...3'                                                  1101
```

FIG. 3. Primary structure of cloned pancreatic glucagon mRNA and the amino acid sequence of preproglucagon. Glucagon and two glucagon-like peptides (amino acid residues 33–61, 72–108, and 126–158, respectively) are underscored. Basic dipeptides that are potential proteolytic processing sites are enclosed in boxes. The sequence of glicentin (residues 1–69) is indicated by vertical arrows. Underscored A-A-T-A-A-A sequences in the 3′-untranslated region are characteristic sites involved in polyadenylylation of eukaryotic mRNAs, but only the site at nucleotides 1,076–1,081 is believed to be utilized.

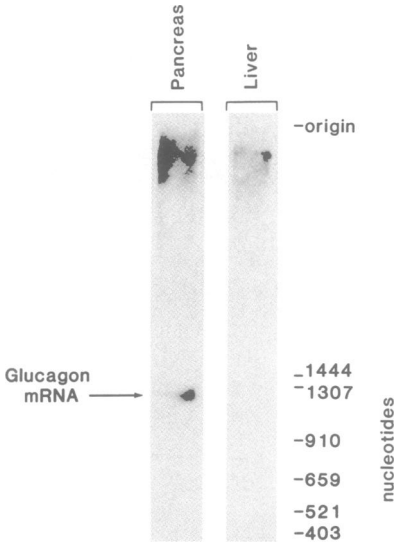

FIG. 4. Hybridization of ^{32}P-labeled glucagon cDNA insert to pancreas poly(A)$^+$ RNA. Ten micrograms each of fetal bovine pancreas and liver (as control) poly(A)$^+$ RNAs was subjected to electrophoresis on 1.5% agarose gels in the presence of 10 mM CH_3HgOH and transferred to nitrocellulose. Hybridization with ^{32}P-labeled-glucagon cDNA insert (specific activity = 10^8 cpm/μg) was at 42°C in 0.75 M NaCl/0.075 M Na citrate/50% formamide/10% dextran sulfate. Filters were washed and dried, and bands were visualized by autoradiography. Size markers, *Alu* I and *Taq* I restriction endonuclease-digested plasmid pBR322 DNA, were run in a parallel lane and visualized by staining with ethidium bromide prior to transfer.

DISCUSSION

Our finding of a mammalian pancreatic preproglucagon of 180 amino acids containing the sequence of glicentin and two additional glucagon-related peptides is surprising. It had been demonstrated previously by Lund *et al.* (28) that anglerfish preproglucagon consisted of 124 amino acids, shared little homology with glicentin (other than the glucagon moiety), and contained only two glucagon-related peptides. This divergence in prohormone structure differs from that observed for proinsulin and prosomatostatin (51, 52), whose structural organizations are conserved between fish and mammals. The 56-amino acid difference between the bovine and fish preproglucagons is accounted for by the seven additional amino acids in the bovine sequence between glucagon and GLP-I and the 49 carboxyl-terminal amino acids that compose GLP-II and its flanking region. The 66% homology between the second glucagon-like peptides (bovine GLP-I and fish glucagon-related COOH peptide) approaches the 69% conservation between the glucagons of these species. The size of the signal peptides (20 amino acids) is the same, and they share 25% sequence homology. In contrast, bovine–fish homology in the region comprising amino acid residues 1–30 of proglucagon (the GRPP) is only 13%. The sequences joining fish and bovine glucagons (amino acids 64–69 in bovine) and the glucagon-like peptides that follow are not conserved between the two species in size or sequence and may serve only as spacers.

Based upon the amino acid sequence of preproglucagon that we have deduced from the nucleotide sequence of its cloned cDNA, we propose a scheme for the post-translational processing of pancreatic preproglucagon (Fig. 5). Our processing scheme is consistent with the results of Patzelt *et al.* (9), who analyzed glucagon processing in cultured rat islets, and Moody *et al.* (15, 53), who determined that a peptide with glicentin-like immunoreactivity (and lacking glucagon-like immunoreactivity) was secreted synchronously with glucagon in the perfused porcine pancreas.

We suggest that the initial translation product of ≈21 kDa contains a 20-amino acid signal peptide that is cotranslationally cleaved to produce the 18-kDa proglucagon. This fragment is cleaved at the Lys-Arg sequence (residues 31 and 32) in glicentin, releasing the GRPP (residues 1–30) and the 13-kDa precursor. Cleavage at the Lys-Arg sequence (residues 70 and 71), which follows the octapeptide extension at the carboxyl terminus of glucagon in the 13-kDa precursor, produces the 4.5-kDa precursor and a 10-kDa fragment containing GLP-I and GLP-II. Proteolytic processing at the Lys-Arg sequence (residues 62 and 63) of the 4.5-kDa precursor results in the 3.5-kDa mature glucagon. The fate of the 10-kDa fragment containing GLP-I and GLP-II is unknown; these peptides apparently do

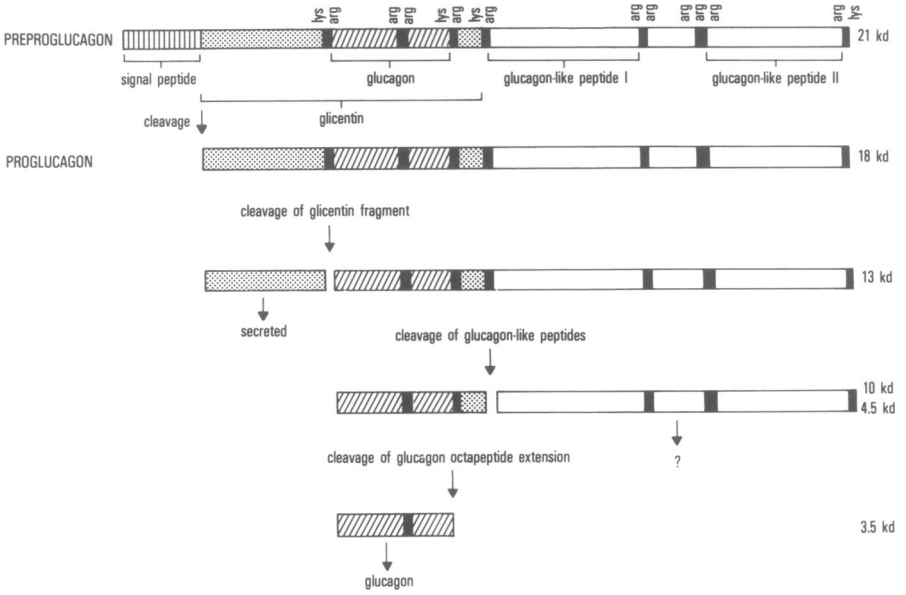

FIG. 5. Proposed scheme for pancreatic preproglucagon processing. This biosynthetic pathway for the processing of pancreatic preproglucagon is based upon our deduced precursor amino acid sequence and the results of Patzelt *et al.* (9) and Moody *et al.* (15, 53). Estimation of the M_r of the precursors are from Patzelt *et al.* (9). kDa, kilodaltons.

not share immunological determinants with glucagon or glicentin, and therefore the pathway of their processing was not followed in studies utilizing antibodies specific for the latter polypeptides (9). Thus, it is not known whether the two possible Arg-Arg proteolytic processing sites (residues 109 and 110 and residues 124 and 125) are utilized. The Arg-Arg sequence within glucagon (residues 49 and 50) is known not to be cleaved in the pancreas. It is possible that the variety of possible processing sites mediates a tissue-specific expression of the glucagon gene. At least one of the possible processing sites (residues 31 and 32) seems to be cleaved in a tissue-specific manner, resulting in the production of glicentin in the intestine and GRPP and glucagon in the pancreas (14, 15, 53).

Glucagon is part of a family of structurally related peptides that includes secretin, vasoactive intestinal peptide, gastric inhibitory peptide, peptide HI-27, growth hormone-releasing factor, and prealbumin. GLP-I and GLP-II are similar but not identical to the other members of this peptide family. The structure of the glucagon mRNA suggests a tandem triplication of the primary glucagon sequence. No function for GLP-I or GLP-II is known.

The number of glucagon-coding sequences detected in our cDNA library (25 of 3,100) and the 10-fold enrichment of the mRNA cloned suggests that glucagon mRNA constitutes ≈0.1% of the mRNA molecules in second-trimester fetal bovine pancreas. This estimated value compares with an ≈10% abundance of insulin in a similar mRNA population (32).

Following submission of this manuscript, the sequence of hamster preproglucagon was reported (54). The structures of bovine and hamster preproglucagons are highly conserved.

L.C.L. is a recipient of a Rosalie B. Hite Predoctoral Fellowship. C.J.S. is a National Institutes of Health Postdoctoral Fellow (5 F32 AM 06571). This research was supported by grants from the KROC Foundation, Robert A. Welch Foundation, U.S. Public Health Service (CA16672), and the Redfern Fund.

1. Sundby, F. (1976) *Metabolism* **25,** Suppl. 1, 1319–1321.
2. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci.* **343,** 1–16.
3. Rigopoulou, D., Valverde, I., Marco, J., Faloona, G. R. & Unger, R. H. (1970) *J. Biol. Chem.* **245,** 496–501.
4. Noe, B. D. & Bauer, G. E. (1971) *Endocrinology* **89,** 624–651.
5. Noe, B. D. & Bauer, G. E. (1973) *Proc. Soc. Exp. Biol. Med.* **142,** 210–213.
6. Tung,, A. K. & Zeregu, F. (1971) *Biochem. Biophys. Res. Commun.* **45,** 387–395.
7. Hellerstrom, C., Howell, S. L., Edwards, J. C. & Andersson, A. (1972) *FEBS Lett.* **27,** 97–101.
8. Hellerstrom, C., Howell, S. L., Edwards, J. C., Andersson, A. & Ostenson, C. (1974) *Biochem. J.* **140,** 13–23.
9. Patzelt, C., Tager, H. S., Carroll, R. J. & Steiner, D. F. (1979) *Nature (London)* **282,** 260–266.
10. Tager, H. S. & Steiner, D. F. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2321–2325.
11. Valverde, I., Rigopoulou, D., Marco, J., Faloona, G. R. & Unger, R. H. (1970) *Diabetes* **19,** 614–623.
12. Srikant, C. B., McCorkle, K. & Unger, R. H. (1976) *Metabolism* **25,** Suppl. 1, 1403–1404.
13. Valverde, I., Villanueva, M. L., Lozano, I. & Marco, J. (1974) *J. Clin. Endocrinol. & Metab.* **39,** 1090–1098.
14. Thim, L. & Moody, A. J. (1981) *Regulatory Peptides* **2,** 139–150.
15. Thim, L. & Moody, A. J. (1982) *Biochim. Biophys. Acta* **703,** 134–141.
16. Mutt, V., Jorpes, J. E. & Magnusson, S. (1970) *Eur. J. Biochem.* **15,** 513–519.
17. Brown, J. C. & Dryburgh, J. R. (1971) *Can. J. Biochem.* **49,** 255–261.
18. Mutt, V. & Said, S. I. (1974) *Eur. J. Biochem.* **42,** 581–589.
19. Jornvall, H., Carlstrom, A., Pettersson, L., Jacobsson, B., Persson, M. & Mutt, V. (1981) *Nature (London)* **291,** 261–262.
20. Tatemoto, K. & Mutt, V. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6603–6607.
21. Spiess, J., Rivier, J., Thorner, M. & Vale, W. (1982) *Biochemistry* **21,** 6037–6040.
22. Sokal, J. E. (1973) in *Methods in Investigative and Diagnostic Endocrinology*, eds. Berson, S. A. & Yalow, R. S. (Elsevier, New York), pp. 901–913.
23. Hers, H. G. (1976) *Annu. Rev. Biochem.* **45,** 167–188.
24. Lefebvre, P. J. & Luyckx, A. S. (1979) *Diabetologia* **16,** 347–354.
25. Lund, P. K., Goodman, R. H. & Habener, J. F. (1981) *J. Biol. Chem.* **256,** 6515–6518.
26. Lund, P. K., Goodman, R. H., Jacobs, J. W. & Habener, J. F. (1980) *Diabetes* **29,** 583–586.
27. Shields, D., Warren, T. G., Roth, S. E. & Brenner, M. J. (1981) *Nature (London)* **289,** 511–514.
28. Lund, P. K., Goodman, R. H., Dee, P. C. & Habener, J. F. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 345–349.
29. Matteucci, M. D. & Caruthers, M. H. (1981) *J. Am. Chem. Soc.* **103,** 3185–3191.
30. Beaucage, S. L. & Caruthers, M. H. (1981) *Tetrahedron Lett.* **22,** 1859–1862.
31. Lomedico, P. T. & Saunders, G. F. (1976) *Nucleic Acids Res.* **3,** 381–392.
32. Frazier, M. L., Montagna, R. A. & Saunders, G. F. (1981) *Biochemistry* **20,** 367–371.
33. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
34. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5202.
35. Mevarech, M., Noyes, B. E. & Agarwal, K. L. (1979) *J. Biol. Chem.* **254,** 7472–7475.
36. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–559.
37. Murphy, E. C., Jr., Kopchick, J. J., Watson, K. F. & Arlinghaus, R. B. (1978) *Cell* **13,** 359–369.
38. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
39. Land, H., Grez, M., Hauser, H., Lindenmaier, W. & Schutz, G. (1981) *Nucleic Acids Res.* **9,** 2251–2266.
40. Dagert, M. & Ehrlich, S. D. (1979) *Gene* **6,** 23–28.
41. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3961–3965.
42. Agarwal, K. L., Brunstedt, J. & Noyes, B. E. (1981) *J. Biol. Chem.* **256,** 1023–1028.
43. Sanger, F. S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
44. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9,** 309–321.
45. Bailey, J. M. & Davidson, N. (1976) *Anal. Biochem.* **70,** 75–85.
46. Weinstock, R., Sweet, R., Weise, M., Cedar, H. & Axel, R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1299–1303.
47. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
48. Blobel, G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1496–1500.
49. Lewin, G. (1975) *Cell* **4,** 11–20.
50. Proudfoot, N. J. & Brownlee, G. G. (1976) *Nature (London)* **263,** 211–214.
51. Shen, L. P., Pictet, R. L. & Rutter, W. J. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4575–4579.
52. Chan, S. J., Emdin, S. O., Kwok, S. C. M., Kramer, J. M., Falkmer, S. & Steiner, D. F. (1981) *J. Biol. Chem.* **256,** 7595–7602.
53. Moody, A. J., Holst, J. J., Thim, L. & Jensen, S. L. (1981) *Nature (London)* **289,** 514–516.
54. Bell, G. I., Santerre, R. F. & Mullenbach, G. T. (1983) *Nature (London)* **302,** 716–718.