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SPECIFIC DETECTION OF HUMAN AND RABBIT GLUCAGON mRNA USING A SYNTHETIC OLIGODEOXYNUCLEOTIDE

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SUMMARY:

A unique 14 base oligodeoxynucleotide dTTCATCAGCCACTG complementary to glucagon mRNA was deduced from the amino acid sequence of the hormone (residues 24-28; GLN-TRP-LEU-MET-ASN). The oligonucleotide specifically hybridized to RNA from rabbit pancreas and human pancreatic islet cells. No detectable hybridization was observed with either yeast or rat liver RNA. The melting temperature of the hybrids was 50 ± 5°C indicating no significant mismatch for human or rabbit glucagon mRNA. Hybridization kinetics followed a single pseudofirst-order reaction ($Cot_{0.5}$ = 2.5 x 10^{-4} M sec). From the extent of reaction at completion there are a minimum of 43 fmol glucagon mRNA/mg RNA (total pancreas).

INTRODUCTION

Glucagon is the major polypeptide hormone produced by the alpha cells of the Islets of Langerhans. It has been postulated to have a role in the induction of diabetes (1) as well as its well known function in the regulation of blood glucose and insulin secretion. In attempting to determine the factors regulating glucagon biosynthesis, it is necessary to isolate and purify glucagon mRNA. We have chosen to use the strategy devised by Wu (2) and Agarwal and coworkers (3) in which a short segment of the amino acid sequence is used to deduce the sequence of a unique oligodeoxynucleotide complementary to the mRNA. This oligonucleotide is then used as a sensitive and specific probe for the mRNA (4-6).

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1. Abbreviations: Tris - tris(hydroxymethyl) amino methane; SDS - sodium lauryl sulfate; A+RNA - poly A containing mRNA; PB - sodium phosphate buffer (ph 6.8).

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Briefly we have synthesized the 14 base oligonucleotide dTTCATCAGCCAC-TG which complements the segment of glucagon mRNA coding for the amino acids ^{24}Gln-^{25}Trp-^{26}Leu-^{27}Met-^{28}Asn. We find that the 14-mer rapidly and specifically hybridizes pancreas A+ mRNA.[1] The hybrids appear to be nearly perfect matches as judged by thermal denaturation profiles, and reaction kinetics. We conclude that the 14-mer is a specific and sensitive probe for glucagon mRNA sequences in heterogeneous populations of RNA.

Materials. The oligodeoxynucleotide dTTCATCACCCACTG was chemically synthesized by Collaborative Research using the diester approach and its nucleotide sequence confirmed by two dimensional homochromatography (7). Oligo (dT) - cellulose (Type T-3) was purchased from Collaborative Research. Aminobenzyloxymethyl paper was obtained from S & S. Nuclease-free T4 polynucleotide kinase was a product of P & L Laboratories and used for 5'-labeling of oligonucleotides (9, 17) subsequently analyzed on polyacrylamide gels (18). Reverse transcriptase (RNA-dependent DNA polymerase) from avian myeloblastosis virus was provided by J.W. Beard (Life Sciences, Inc.) [γ-^{32}P] ATP was a gift from Kaaren Janssen (Specific activity > 6000 Ci/mmol).

Isolation of Pancreas A$^+$ RNA - Rabbit pancreas were obtained from freshly killed rabbits and quick frozen on dry ice until ready for processing. Samples of human pancreas islets were obtained from autopsy samples and similarly treated. Total RNA was obtained by a modification of the guanidine HCl extraction procedure of Cox (10) using glassware which had been previously baked or treated with diethylpyrocarbonate to remove any traces of RNase. A$^+$RNA was isolated on oligo-dT-cellulose (11). All fractions of RNA were assayed for purity and intactness by electrophoresis in 1.5% agarose gels in the presence of 4 mM CH_3 HgOH (12) and translatability in the wheat embryo *in vitro* system (13, 16). *In vitro* translation products were monitored on SDS - 9% polyacrylamide gels as described by Laemmli (14). The RNA preparations used here stimulated the incorporation of ^{35}S-methionine into large protein products 3 to 5 fold over background.

Direct Immobilization of RNA on DBM Paper - RNA samples (1-50μg) were dissolved in < 20μl 0.1M sodium acetate buffer pH 4 and spread on 1 x 1 cm squares of DBM paper freshly prepared as described by Alwine et al (8). The squares were dried at 60°C then rewet with H_2O and air dried. The degree of RNA binding was measured by soaking the squares for one hour in 0.1M NaCl then checking A of the solution.

Hybridization with the Radiolabeled 14 mer - Hybridization reactions were carried out at 20°C in 0.5 M PB containing 0.2% SDS. Solutions containing the appropriate amount of RNA and 14 mer (10^6 to 10^7 cpm/μl) were heated to 100°C for 1-2 minutes, then cooled to 20°C and allowed to reanneal. The extent of reaction as a function of time was monitored on an Ultrogel A-44 column (0.7 x 100 cm) in 0.12 M PB/0.1% SDS. Hybridization of the labeled 14 mer to RNA covalently linked to paper was essentially as described for the solution reactions. The sequence excess of 14 mer to its complement in mRNA was estimated to be $\geq$ 300:1. The reaction rates are expressed in terms of equivalent C_0t (19).

Thermal Denaturation of Hybrids - Hybridized labeled 14 mer bound to human islet or rabbit pancreas A+ RNA-paper chips was washed extensively with 0.12 M PB/0.1% SDS until no further counts were eluted. Each chip was then placed in 500 μl 0.12 M PB/0.1% SDS and heated to the appropriate temperature in a thermostated heating block for 10 minutes. The chips were then removed, placed in fresh buffer and counted using Cerenkov radiation excited by ^{32}P. This procedure was repeated at 5°C intervals up to 100°C.

RESULTS AND DISCUSSION

Our approach to the isolation of mRNAs involves the chemical synthesis of oligodeoxynucleotides complementary to the mRNA deduced from favorable, short sequences of amino acids Wu (2). In essence, one scans the known protein sequence looking for amino acid residues specified primarily by a single codon, for example methionine (AUG). In glucagon a favorable amino acid sequence is found in residues 24-28 for which the glucagon mRNA complementary oligodeoxynucleotide sequence dTTCATCAGCCACTG can be deduced as shown in Fig. 1. The rationale for the degenerate codons is as follows. Glutamine is specified in most sequenced mRNAs and cloned DNAs by the codon CAG (e.g., in globin mRNA). Thus the sequence CAG is selected. For asparagine the first two bases are precisely specified. The third base is simply eliminated. For leucine, the chance that C occurs in the first position on a random basis is 4 in 6. Using G gives a 67% chance of being correct (G = C) and a 33% of forming a stable G-T wobble base pair (2). The third position remains in doubt. We base our choice for the third base on the prevalance of third position G (e.g., gastrin mRNA codes leucine with CUG 3 times and CUC one time) in sequenced mRNAs. At worst there is a 33% chance that C is correct on a random basis alone.

The oligodeoxynucleotide dTTCATCAGCCACTG was synthesized by the phosphodiester method, (3) purified by reverse phase chromatography, and sequenced

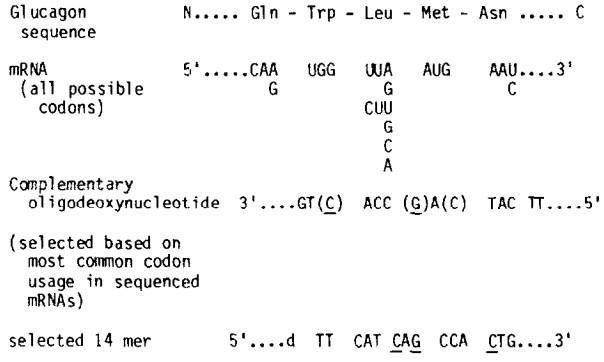

Fig. 1. The Deduction of the Specific Sequence of an Oligodeoxynucleotide Complementary to Glucagon mRNA.

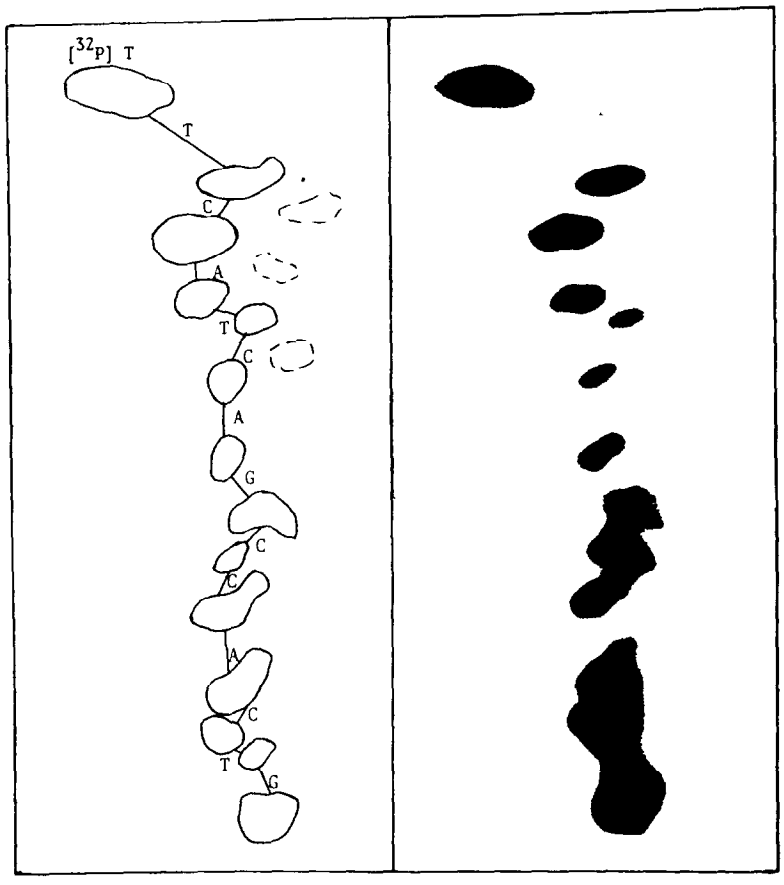

Fig. 2. Two Dimensional Mobility Shift Chromatography of the Oligonucleotide dTTCATCAGCCACTG. Conditions as described in Methods.

using two dimensional homochromatography. As shown in Fig. 1 the product oligonucleotide had the sequence dTTCATCAGCCACTG. The radioautograms were intentionally overexposed to show minor spots.

^{32}P-labeled 14 mer specifically hybridized to human islet or rabbit pancreas A^+ RNA covalently bound to paper chips. The extent of reaction was directly proportional to the amount of RNA (Fig. 2A). Control reactions utilizing yeast RNA and rat liver RNA under the same conditions yielded no detectable hybrids, indicating specific hybridization with pancreas RNA. This result was confirmed by solution hybridization between human islet RNA and ^{32}P-14 mer using gel-filtration as shown in Fig. 3B. Similar data were obtained for rabbit pancreas total RNA which bound 43 fmol 14-mer/mg RNA. We estimate the detectable level of glucagon mRNA to be less than 0.1 fmol.

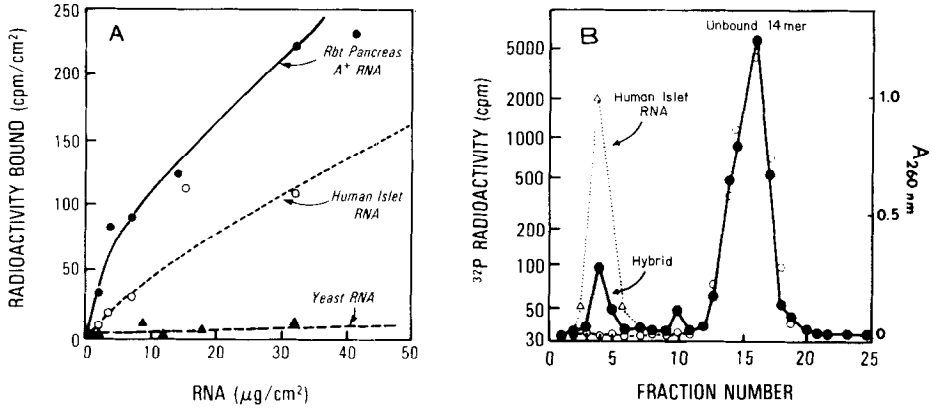

Fig. 3A. Specific Hybridization of 14 mer to Pancreas RNA Covalently Bound to Paper. ^{32}P labeled 14 mer (SA - 5.3 x 10^4 dpm/pmol) was hybridized to RNA - paper samples in 0.5 M Na phosphate (pH 6.8) containing 0.2% SDS for 60 hours at 20°C background binding (85 cpm) has been subtracted.

Fig. 3B. Ultrogel A-44 Chromatography of 14 mer Hybridized to Human Islet RNA. Human islet RNA (100 μg) was hybridized to ^{32}P-labeled 14 mer (6 nM) in solution for 72 hours at 20°C, then chromatographed on Ultrogel A-44 in 0.12 M Na phosphate (pH 6.8) contain 0.2% SDS. RNA was monitored by A_{260} (△·····△); Human islet RNA x 14 mer (●—●—●); 14 mer alone (○—○—○).

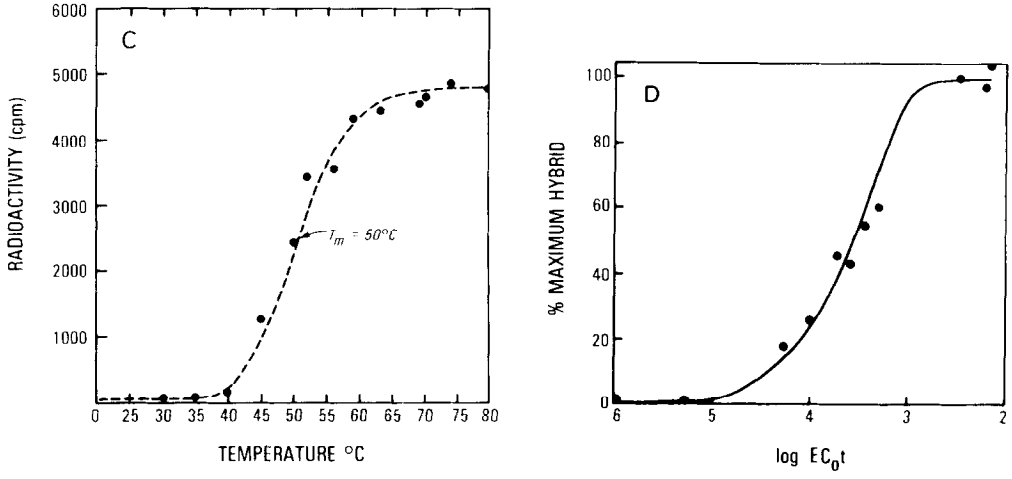

Fig. 3C. Thermal Denaturation Profile of 14 mer: Rabbit Pancreas A+ mRNA-paper Hybrids. Denaturation as described in Methods. In 0.12 M Na phosphate (pH 6.8) containing 0.1% SDS.

Fig. 3D. Kinetics of Hybridization of 14 mer to Rabbit Pancreas A+ mRNA-paper. Conditions as in Fig. 2A [14 mer] = 6.1 nM. Curve is theoretical first order reaction ($K = 2.8 \times 10^3\ M^{-1}\ sec^{-1}$).

The thermal denaturation profiles for ^{32}P-14 mer:RNA-paper hybrids are shown in Fig. 3C. The hybrids for rabbit pancreas A+ RNA had a T_m of 50 ± 5°C. Identical results were obtained for human islet A+ RNA:14 mer hybrids. The expected T_m for a perfectly matched 14 mer duplex with a 50% GC content is

about 43°C in 0.18 M Na^+ (19) although sequence isomers could melt as much as 10°C higher (20). Since a single mismatch would be expected to lower T_m by about 10°C (21), the observation of a melting temperature 7°C above the expected T_m (43°C) precludes significant base mismatching. It has been previously observed that RNA:DNA hybrids bound to solid support are significantly more stable than their corresponding solution congeners (22, 23). This observation could account for the higher than expected melting temperature of the 14 mer:RNA hybrids.

The reaction of the 14 mer with rabbit pancreas A+ RNA paper chips followed ideal pseudo-first order kinectics indicating a single class of reactants as shown in Fig. 3D. The curve is a theoretical curve obtained from a linear regression analysis of a first order plot. The $ECot_{1/2}$ for the reaction was 2.5×10^{-4} M sec ($K = 2.8 \times 10^{3} M^{-1} Sec^{-1}$).

The kinetics of hybridization are consistent with the reaction of a single species of RNA with the 14 mer. The rate constant for the reaction is very close to the recorded rate for short oligomers with immobilized Øx174 DNA ($K = 1.3 \times 10^{3} M^{-1} sec^{-1}$) (21). The difference between the rates probably lies in the temperature of hybridization which was 8°C lower in the case of the ØX174 DNA - oligonucleotide interaction (21). In both cases the rate of reaction is approximately 10 fold lower than the rate which would be predicted from the kinetics of E. coli DNA reassociation when corrected for fragment length and complexity (19). The reason for this discrepancy is not clear.

These data are consistent with the idea that the synthetic oligodeoxynucleotide reacts specifically with glucagon mRNA sequences. The chances of another perfectly matched 14 base sequence occurring at random is 4^{14} or 1 in 2.7×10^{8}. Since some proteins appear to be derived from one another by gene duplication, it is possible that one 14 mer sequence may be present in more than one mRNA. In order to determine the exact nature of the reacting mRNA, it will be necessary to synthesize and sequence cDNA specifically primed by the oligomer. These studies are currently underway.

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