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Use of Oligodeoxynucleotide Primers To Determine Poly(adenylic acid) Adjacent Sequences in Messenger Ribonucleic Acid. 3′-Terminal Noncoding Sequence of Bovine Growth Hormone Messenger Ribonucleic Acid†

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ABSTRACT: Twelve synthetic oligodeoxynucleotide primers of the general sequence d(pT_8-N-N′) were tested in a reverse transcriptase reaction for specific initiation of complementary deoxyribonucleic acid (cDNA) synthesis at the poly(adenylic acid) junction of a messenger ribonucleic acid (mRNA) template. Only the sequence d(pT_8-G-C) functioned as a specific primer of cDNA synthesis with an enriched fraction of bovine growth hormone mRNA from the anterior pituitary gland and produced unique fragments in a dideoxy sequencing reaction. The nucleotide sequence obtained by this method extended into the protein coding region of bovine growth hormone mRNA and was confirmed by chemical sequencing of the cDNA initiated with [5′-^{32}P]d(pT_8-G-C). The 3′-untranslated region of bovine growth hormone mRNA is 104 nucleotides in length and contains regions of significant homology with both rat and human growth hormone mRNAs, including the region surrounding the common AAUAAA hexanucleotide. The method presented here for selection of the d(pT_8-N-N′) primer complementary to the poly(A) junction of mRNA is of general applicability for nucleotide sequence analysis of partially purified mRNAs.

An understanding of the biosynthesis and function of a specific mRNA requires a knowledge of its primary structure. Advances in both DNA and RNA nucleotide sequence analysis in the past several years have greatly stimulated studies of specific genes and gene transcripts at the molecular level. The development of two rapid DNA sequencing methods involving termination of growing DNA chains (Sanger et al., 1977) and chemical cleavage of terminally labeled DNA (Maxam & Gilbert, 1977) has contributed most significantly to DNA structural analysis of cloned gene sequences. Occassionally, however, it is possible to isolate significant quantities of specific mRNA molecules from which sequence information may be obtained directly without prior cloning of the mRNA sequences. Indeed, a number of investigators have now adapted

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the rapid DNA sequencing technology to determine the primary structure of mRNA molecules directly.

The dideoxy chain termination method for DNA sequencing (Sanger et al., 1977) has recently been modified for use with reverse transcriptase directed cDNA synthesis. Zimmern & Kaesberg (1978) specifically primed cDNA synthesis at the poly(A)[1] junction of encephalomyocarditis virus mRNA using the oligonucleotide $d(pT_7)rC$. Their analysis confirmed and extended the previously known 3′-terminal 26 nucleotides of this viral mRNA. Similarly, McGeoch & Turnbull (1978) determined a sequence of 205 nucleotides adjacent to the poly(A) tail of vesicular stomatitis virus N protein mRNA using $d(pT_8\text{-}C)$ as a primer for cDNA synthesis in the presence of dideoxy chain terminators. The complementary $d(pT_8\text{-}N)$ primer sequence for cDNA synthesis was determined by using $d(pT_{10})$ to initiate transcription of the template in the absence of TTP (Schwartz et al., 1977). Only those oligo(dT) primers that hybridized at the poly(A) junction of the viral mRNA template were able to initiate reverse transcription under these conditions. In this manner, the G residue adjacent to the poly(A) sequence was determined.

Other partial sequences of mRNAs have been determined directly by adapting the dideoxy chain termination method for use with specific synthetic oligonucleotides complementary to internal regions of the mRNA (Hamlyn et al., 1978) and small restriction fragments (Bina-Stein et al., 1979) as primers for cDNA synthesis. In yet another adaptation of the rapid DNA sequencing technology, Noyes et al. (1979) utilized a 5′-^{32}P-labeled oligodeoxynucleotide probe complementary to a unique amino acid codon sequence of hog gastrin mRNA to prime cDNA synthesis. The resultant gastrin-specific cDNA was subsequently analyzed by the chemical degradation method of DNA sequencing developed by Maxam & Gilbert (1977).

In the above analyses, previous sequence information was available for portions of the mRNA prior to selection of a specific oligonucleotide primer for cDNA synthesis. We present here a method for obtaining nucleotide sequence information for any partially purified poly(A)-containing mRNA without previous sequence analysis by some alternate method. The technique involves phased priming at the poly(A) junction of the mRNA with oligodeoxynucleotide primers of the general sequence $d(pT_8\text{-}N\text{-}N')$. The addition of a second nucleotide to the three possible $d(pT_8\text{-}N)$ sequences greatly enhances the selectivity of the cDNA priming reaction, thus facilitating the analysis of partially purified mRNAs. Growth hormone (GH) mRNA, enriched from bovine anterior pituitary poly(A) RNA, was tested in a dideoxy chain termination reaction with the 12 possible $d(pT_8\text{-}N\text{-}N')$ sequences to determine the specific primer complementary to the poly(A)-adjacent nucleotides. Of the 12 oligonucleotide sequences, only $d(pT_8\text{-}G\text{-}C)$ functioned as a specific primer for initiation of bovine GH cDNA synthesis and produced unique fragments in the dideoxy sequencing reaction. This method of phased priming at the poly(A) junction of the mRNA template has allowed us to determine the complete 3′-noncoding sequence of bovine GH mRNA by both the chain termination and chemical cleavage methods of DNA sequencing.

Materials and Methods

Materials. Reverse transcriptase from avian myeloblastosis virus was generously provided by Dr. J. W. Beard (Life Sciences, Inc., St. Petersburg, FL). T_4 polynucleotide kinase was purchased from Bethesda Research Labs, and *Escherichia coli* alkaline phosphatase was obtained from Worthington. [α-^{32}P]dCTP (400 Ci/mmol) was from Amersham, and [γ-^{32}P]ATP (1000–3000 Ci/mmol) was from New England Nuclear. The 2′,3′-dideoxynucleoside triphosphates and the deoxynucleoside triphosphates were purchased from P-L Biochemicals. The dideoxy compounds were used as supplied, and the deoxynucleoside triphosphates were purified by DEAE-cellulose chromatography as described by Brown & Smith (1977).

Purification of Bovine Growth Hormone mRNA. Polysomal RNA from fresh or liquid nitrogen frozen bovine anterior pituitary glands was obtained by magnesium precipitation essentially as described by Palmiter (1974). The RNA was subsequently chromatographed on oligo(dT)–cellulose to obtain the poly(A) mRNA fraction as previously described (Nilson et al., 1979). GH mRNA was enriched by a modification of the approach described by Nilson et al. (1979). The details of this procedure will be described elsewhere (Nilson et al., 1980). Briefly, GH mRNA was obtained from poly(A) RNA by sucrose density gradient centrifugation on 5–20% linear sucrose gradients. The GH mRNA fractions were identified by in vitro translation, pooled, ethanol precipitated, and centrifuged on identical sucrose gradients one or two additional times. The final bovine GH mRNA preparation was estimated to be approximately 70–80% pure by in vitro translation, with the major contaminant being prolactin mRNA.

Synthesis of Oligodeoxynucleotide Primers. The 12 oligodeoxynucleotides of the general sequence $d(pT_8\text{-}N\text{-}N')$ and the three $d(pT_8\text{-}N)$ sequences were enzymatically synthesized by using *E. coli* polynucleotide phosphorylase. The complete conditions for the synthetic reactions and the oligonucleotide characterizations are described by Gillam & Smith (1980).

Screening of Oligodeoxynucleotide Primers for Specific Initiation of cDNA Synthesis. The 12 synthetic oligodeoxynucleotide primers with the general sequence $d(pT_8\text{-}N\text{-}N')$ were screened by a chain termination reaction for specific initiation of cDNA synthesis with partially purified bovine GH mRNA. The reverse transcriptase directed cDNA synthesis using GH mRNA as a template was conducted in the presence of the chain terminator ddTTP. Each reaction contained 0.03 μg of enriched bovine GH mRNA and was incubated 10 min at 37 °C in a 5-μL volume with 60 mM Tris (pH 8.3), 75 mM NaCl, 7.5 mM $MgCl_2$, 25 mM DTT, 2.5 μM [α-^{32}P]dCTP (330 Ci/mmol), 50 μM each of dATP and dGTP, 2.5 μM each of TTP and ddTTP, 17 μg/mL $d(pT_8\text{-}N\text{-}N')$, and 270 units/mL reverse transcriptase. The reactions were performed in capillary tubes and incubated by placing the capillaries in a 6 × 50 mm siliconized test tube in a water bath. At the end of the incubation period, 0.25 μL of a mixture containing 2.5 mM each of the four dNTPs was added to the reaction mixtures in the capillaries, and synthesis was continued for 5 min at 37 °C. The contents of the capillaries were then emptied into the siliconized test tubes, and the reactions were stopped by the addition of 5 μL of 90% formamide, 25 mM Na_2EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanol. Each sample was heated in the test tubes to 100 °C for 3 min and quickly chilled on ice.

In these screening assays, electrophoresis of the samples (5 μL) was performed on a 12% polyacrylamide–7 M urea slab gel (0.5 × 200 × 400 mm) in 50 mM Tris-borate (pH 8.3) and 1 mM Na_2EDTA buffer for 3 h at 25 W (Sanger &

[1] Abbreviations used: GH, growth hormone; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; ddNTP, 2′,3′-dideoxynucleoside triphosphate; poly(A), poly(adenylic acid); oligo(dT), oligo(deoxythymidine).

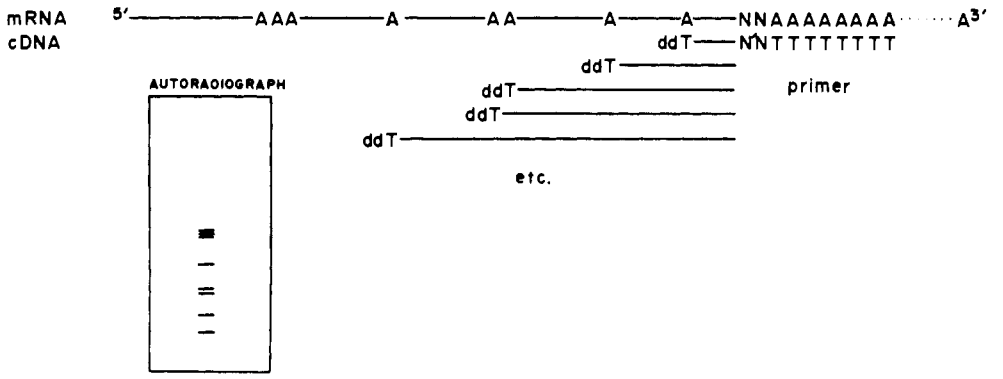

FIGURE 1: Method for determination of the d(pT_8-N-N') oligodeoxynucleotide primer complementary to poly(A)-adjacent nucleotides in mRNA. The above figure illustrates the principle for selection of the specific d(pT_8-N-N') sequence complementary to the two 3'-terminal nucleotides of a mRNA sequence. The reverse transcriptase directed cDNA synthesis is carried out in the presence of one dideoxynucleoside triphosphate such as ddTTP. Chain termination occurs at specific nucleotides in the sequence when cDNA synthesis is initiated by the complementary primer. Gel electrophoresis and autoradiography of the labeled fragments on a sequencing type gel produce a unique and specific pattern of bands.

Coulson, 1978). The gel was fixed in 10% acetic acid for approximately 10 min, rinsed in water, and covered with Saran plastic wrap. Autoradiography of the gel was conducted at room temperature overnight with Kodak NS-5T film.

Dideoxy Sequencing Conditions. The oligodeoxynucleotide complementary to the poly(A) junction of bovine GH mRNA, as determined in the reaction described above, was d(pT_8-G-C). This primer was used to initiate cDNA synthesis on the GH mRNA template in the presence of each of the four dideoxy chain terminators. The individual 5-μL reactions in capillaries contained 0.08 μg of partially purified bovine GH mRNA, 60 mM Tris (pH 8.3), 75 mM NaCl, 7.5 mM $MgCl_2$, 25 mM DTT, 2.5 μM [α-^{32}P]dCTP (330 Ci/mmol), 2.5 μM each of the appropriate ddNTP and its counterpart dNTP, 50 μM of the other two dNTPs, 17 μg/mL d(pT_8-G-C), and 270 units/mL reverse transcriptase. The capillaries containing the reactions were incubated 10 min at 37 °C before the addition of 1 μL of a "cold chase" mix containing 2.5 mM of each dNTP. Incubation was continued for 5 min at 37 °C, and the synthesis was terminated by the addition of the dye mixture as described above. The reactions were then heated at 100 °C for 3 min and quickly chilled on ice. The samples were divided into 5-μL aliquots and electrophoresed on 8, 12, and 20% polyacylamide–7 M urea slab gels. The sequencing gels were treated and autoradiographed as described above.

Chemical Sequencing Conditions. The oligodeoxynucleotide, d(pT_8-G-C), was dephosphorylated and labeled with ^{32}P at the 5' end for use in the chemical sequencing reactions of Maxam & Gilbert (1977). The dephosphorylation reaction with *E. coli* alkaline phosphatase was performed in 45 mM ammonium formate for 3 h at 37 °C (Montgomery et al., 1978). The reaction was stopped by the addition of EDTA. The sample was boiled for 5 min and then applied to Whatman No. 40 paper. Descending paper chromatography was conducted for approximately 35 h in 1-propanol–NH_4OH–H_2O (55:10:35). The oligodeoxynucleotide was located by UV absorption and eluted from the paper with water adjusted to pH 10 with NH_4OH. Dephosphorylated d(pT_8-G-C) was then labeled with ^{32}P at the 5' end using T_4 polynucleotide kinase and [γ-^{32}P]ATP. Approximately 120 pmol of d(pT_8-G-C) was incubated with an equimolar amount of [γ-^{32}P]ATP (1000–3000 Ci/mmol) according to van de Sande et al. (1973). The reaction was stopped with 0.25 M EDTA, and the sample was applied directly to a Sephadex G-25 column (0.7 × 26 cm). The ^{32}P-labeled primer was separated from the unreacted [γ-^{32}P]ATP at a flow rate of approximately 0.25 mL/min with 50 mM NH_4HCO_3. The fractions containing the labeled primer were pooled and lyophilized to dryness.

The ^{32}P-labeled d(pT_8-G-C) (approximately 5 × 10^5 cpm/pmol) was used to specifically initiate cDNA synthesis with bovine GH mRNA. A 175-μL reaction mixture contained 8 μg/mL RNA, 200 μM of each dNTP, 50 mM Tris (pH 8.3), 60 mM NaCl, 6 mM $MgCl_2$, 20 mM DTT, 0.7 pmol/μL [^{32}P]d(pT_8-G-C) primer, and 270 units/mL reverse transcriptase. Incubation was for 1 h at 37 °C. The synthesis was stopped with EDTA, and the reaction mixture was applied to a Sephadex G-200 column (0.7 × 26 cm). The excluded peak of [^{32}P]cDNA was separated from the free ^{32}P-labeled primer with 50 mM NH_4HCO_3. The pooled fractions of [^{32}P]cDNA were lyophilized to dryness and analyzed according to the chemical cleavage method of Maxam & Gilbert (1977).

Results

Screening of d(pT_8-N-N') Primers for Specific Initiation of cDNA Synthesis. Phased priming of reverse transcriptase directed cDNA synthesis at the poly(A) junction of mRNA results in a cDNA with a unique 5' end required for sequence analysis. This approach has been utilized in limited instances where the base or bases adjacent to the poly(A) tract of the mRNA had been previously determined in some other manner (Zimmern & Kaesberg, 1978; McGeoch & Turnbull, 1978). In this study, we have designed a method to screen the 12 oligodeoxynucleotides of the general sequence d(pT_8-N-N') for specific initiation of cDNA synthesis on a mRNA template when the sequence at the poly(A) junction is unknown. The technique involves a modification of the dideoxy chain termination method for DNA sequencing (Sanger et al., 1977).

The principle of our primer screening method is illustrated in Figure 1. Specific initiation of cDNA synthesis is obtained by base pairing of the primer to the poly(A) junction of the mRNA template. A reverse transcriptase reaction is performed in the presence of one dideoxy chain terminator, such as ddTTP. Specific priming of cDNA synthesis is then analyzed on a sequencing-type gel (Sanger & Coulson, 1978). The detection of unique bands by autoradiography identifies the oligodeoxynucleotide complementary to the 3' terminus of the mRNA.

We have used this method to analyze the 3' terminus of GH mRNA. GH is a major anterior pituitary protein, and its mRNA constitutes a large fraction of the total mRNA obtained from this tissue (Nilson et al., 1979). An enriched fraction of bovine GH mRNA isolated by sucrose density gradient centrifugation was screened with the 12 d(pT_8-N-N')

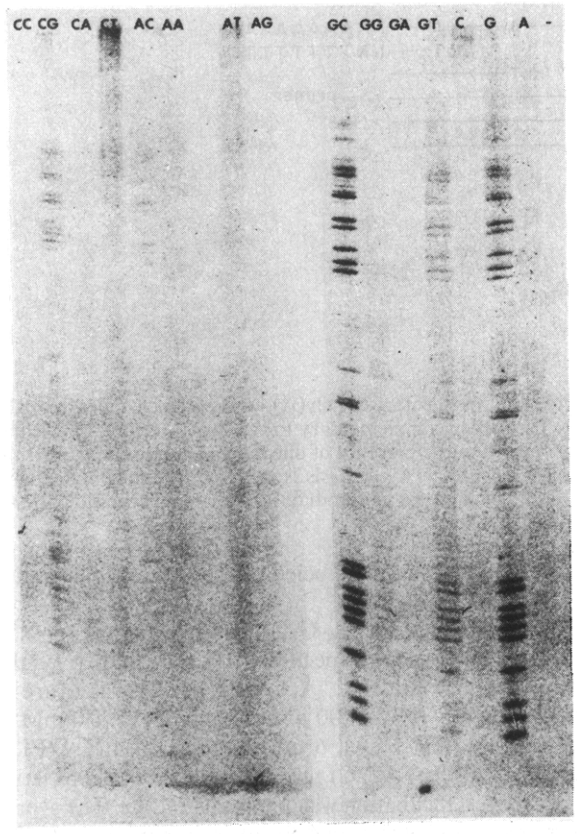

FIGURE 2: Autoradiograph of oligodeoxynucleotide primer screening for specific initiation of bovine GH cDNA synthesis. Twelve synthetic oligonucleotide primers of the sequence d(pT$_8$-N-N′) were screened by a chain termination reaction for specific priming of cDNA synthesis with partially purified bovine GH mRNA. The reactions were carried out in the presence of the chain terminator ddTTP. The labeled fragments were electrophoresed on a 12% polyacrylamide–7 M urea gel to determine which oligonucleotide sequence was complementary to the poly(A) junction of the mRNA template. Initiation of bovine GH cDNA synthesis was specific and most intense with the oligodeoxynucleotide primer d(pT$_8$-G-C) as evidenced by the unique bands synthesized in the presence of this sequence. The fragments produced in the presence of the primer d(pT$_8$-G-T) were less intense and imposed on a background ladder of T residues. This background effect did not reproduce well on this photograph. The lanes of the gel are labeled with the nucleotides of the d(pT$_8$-N-N′) primers. The autoradiograph also shows the three primers of the sequence d(pT$_8$-N) and a control reaction carried out in the absence of a oligonucleotide primer which is indicated by a (–).

primers as described above. No prior sequence information was available to predict the correct oligonucleotide primer for specific initiation of cDNA synthesis on this mRNA template.

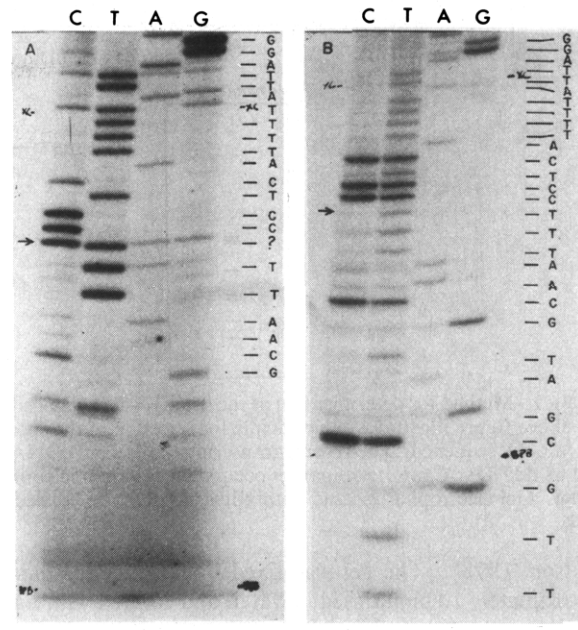

FIGURE 3: Autoradiograph of sequencing gels for bovine GH mRNA. The specific oligodeoxynucleotide primer for bovine GH mRNA, d(pT$_8$-G-C), was used to initiate cDNA synthesis for sequence determination by the dideoxy chain termination and chemical cleavage methods of DNA sequencing. A portion of a dideoxy sequencing gel is shown in panel A. The arrow indicates a nucleotide position where difficulty was encountered in determining the correct nucleotide (see text). Several fragments at the bottom of the gel are nonspecific fragments commonly found at the beginning of dideoxy sequencing patterns. The corresponding nucleotide sequence determined by chemical sequencing of the cDNA is shown in panel B. The arrow indicates the ambiguous nucleotide in the dideoxy sequencing gel. In the chemical sequencing gel, the bases of the d(pT$_8$-G-C) primer are evident. The letters XC and BPB refer to the dye markers xylene cyanol and bromophenol blue, respectively.

Only the sequence d(pT$_8$-G-C) functioned as a specific primer of GH cDNA synthesis as evidenced by the unique and most intense fragments produced in the ddTTP chain termination reaction (Figure 2). This result identifies the 3′-terminal nucleotides adjacent to the poly(A) segment of the mRNA as GC. An identical but very weak banding pattern occurred with the primer d(pT$_8$-G-T) (Figure 2). A faint ladder of T residues imposed on the banding pattern obtained from the primer d(pT$_8$-G-T) may indicate some nonspecific initiation on the poly(A) tail of the mRNA, thus suggesting that d(pT$_8$-G-T) is not a specific primer for bovine GH mRNA. The presence of the T residues at every fragment length on the gel was more apparent with a longer exposure time.

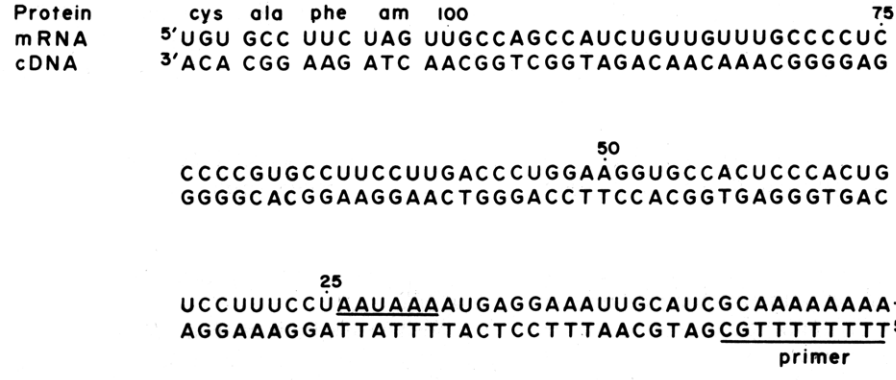

FIGURE 4: Nucleotide sequence of the 3′-terminal noncoding region of bovine GH mRNA. The sequence was determined by chain termination of the cDNA specifically initiated with the primer d(pT$_8$-G-C) and by chemical sequencing of the cDNA initiated by [5′-^{32}P]d(pT$_8$-G-C). The sequence includes the codons for the three carboxyl-terminal amino acids of the growth hormone protein sequence and the amber termination codon UAG. The AAUAAA hexanucleotide commonly found in eucaryotic mRNA sequences is underlined.

Also shown in Figure 2 is the analysis of the three primers d(pT_8-G), d(pT_8-C), and d(pT_8-A). Only d(pT_8-G) primes GH cDNA synthesis, as expected, and produces a banding pattern identical with the one generated in the presence of d(pT_8-G-C). However, the use of the more specific d(pT_8-N-N′) primers to generate a cDNA with a specific 5′ end is more desirable for determination of sequence information with a nonhomogeneous preparation of mRNA. We therefore chose to analyze the 3′-terminal sequence of bovine GH mRNA using the oligodeoxynucleotide d(pT_8-G-C) as a primer for specific initiation of cDNA synthesis.

Sequence Analysis by the Dideoxy Method. The original description of the chain termination method by Sanger et al. (1977) employed DNA polymerase I with a DNA template. Other investigators have since described adaptations of this method for use with reverse transcriptase and mRNA templates (Zimmern & Kaesberg, 1978; McGeoch & Turnbull, 1978; Hamlyn et al., 1978; Bina-Stein et al., 1979). Complementary DNA synthesis by reverse transcriptase is more sensitive to the ddNTP concentration than DNA polymerase I catalyzed reactions (Zimmern & Kaesberg, 1978; MeGeoch & Turnbull, 1978). Consequently, a molar ratio of 1:1 for the dNTP/ddNTP concentration gives good termination for the reverse transcriptase reaction, whereas DNA polymerase I requires a ratio of 1:100 (Sanger et al., 1977). McGeoch & Turnbull (1978) found that using varying ratios of dNTP/ddNTP enabled them to read different lengths of the cDNA sequence. The sequence data presented here for bovine GH mRNA (Figure 3A) were determined by using equimolar concentrations of the dNTP and ddNTPs. However, in several experiments we have found a variation in the sensitivity of reverse transcriptase preparations to the ddNTP concentration (N. L. Sasavage, unpublished experiments).

The sequence obtained for bovine GH mRNA by the dideoxy chain termination technique extended into the protein coding region of the mRNA (Figure 4). The 12 nucleotides containing the termination codon UAG and the codons for the three carboxyl-terminal amino acids of bovine GH, Cys-Ala-Phe (Dayhoff, 1976), provide strong evidence that the 3′-terminal mRNA sequence obtained by initiation of cDNA synthesis with d(pT_8-G-C) is indeed that for bovine GH mRNA.

Difficulty in reading the dideoxy sequencing gels was encountered in several regions. One such region is indicated in Figure 3A. At this point on the mRNA template, termination of cDNA synthesis occurred to approximately the same extent in all four dideoxy reactions, possibly indicating heterogeneity in the nucleotide sequence. In other regions, a variation in the intensity of the bands was used to discriminate the correct nucleotide. Similar observations have been made by other groups and have been attributed to possible sites where reverse transcriptase pauses on the template (Zimmern & Kaesberg, 1978; McGeoch & Turnbull, 1978; Hamlyn et al., 1978; Bina-Stein et al., 1979). Control experiments for bovine GH mRNA run under the same conditions as the sequencing reactions, but without ddNTPs, indicate that natural stops in the cDNA synthesis occur at these ambiguous points in the sequence (data not shown). However, the occurrence of these artifact bands is infrequent and most likely dependent on the inherent primary and secondary structure of the mRNA template. Such results were not a serious problem in reading the sequence.

Sequence Analysis by the Chemical Degradation Method. The dideoxy method of sequence analysis does not provide data on the sequence immediately adjacent to the priming site until the first labeled dNTP is incorporated (Sanger et al., 1977). Our experiments routinely employed [α-^{32}P]dCTP, although any [α-^{32}P]dNTP may be used in the dideoxy sequencing method. Thus, any nucleotides prior to the first G residue of the mRNA following the priming site would not appear on our dideoxy sequencing gels. We have therefore used the Maxam & Gilbert (1977) method of DNA sequence analysis to complete this portion of the bovine GH mRNA sequence and to confirm the nucleotide sequence obtained by the dideoxy sequencing method.

The chemical cleavage sequencing method for DNA requires a ^{32}P label at a defined 3′ or 5′ end of the fragment to be analyzed (Maxam & Gilbert, 1977). The synthesis of cDNA to bovine GH mRNA with [5′-^{32}P]d(pT_8-G-C) presents a unique 5′ terminus for sequence analysis by this method. Recently, Noyes et al. (1979) used a 5′-^{32}P-labeled dodecanucleotide to specifically prime synthesis of gastrin mRNA in the protein coding region. Chemical sequence analysis of specific cDNA fragments isolated from gels provided sequence information corresponding to the amino acid sequence of gastrin. In our experiments, GH-specific cDNA initiated with [5′-^{32}P]d(pT_8-G-C) was separated from free ^{32}P-labeled primer by chromatography on Sephadex G-200 and treated directly to obtain the four base-specific chemical cleavages (Maxam & Gilbert, 1977). The nucleotides of the primer and those immediately adjacent to the primer are clearly visible by this method of analysis (Figure 3B). The sequence information obtained here confirms that deduced by the dideoxy sequence analysis of bovine GH mRNA (Figure 3A). Furthermore, the data resolve the identity of the ambiguous nucleotides obtained with the chain termination method. A single nucleotide was evident at each of five positions in question. This observation clearly rules out sequence heterogeneity in the 3′-noncoding region of bovine GH mRNA.

Discussion

Phased priming of cDNA synthesis at the poly(A) junction of mRNA has provided a successful approach to sequence analysis. Cheng et al. (1976) introduced the technique of specific priming at the 3′ terminus of mRNA to analyze chicken ovalbumin mRNA by T_4 endonuclease IV digestion of the cDNA. With the development of more rapid DNA sequencing methods, several groups adapted the plus and minus gel method of Sanger & Coulson (1975) to analyze cDNA specifically initiated at the poly(A) junction of its template from human and rabbit α-globin (Proudfoot et al., 1977), chicken ovalbumin (Brownlee & Cartwright, 1977), and mouse immunoglobin light-chain (Hamlyn et al., 1977) mRNAs. More recently, the dideoxy chain termination method of DNA sequencing has proven applicable for use with mRNA templates and 3′-terminal-specific oligonucleotide primers (Zimmern & Kaesberg, 1978; McGeoch & Turnbull, 1978). The simplicity of enzymatic synthesis of the 12 oligodeoxynucleotides of the general sequence d(pT_8-N-N′) (Gillam & Smith, 1980) has led us to develop a technique based on the chain termination method (Sanger et al., 1977) for screening an enriched population of poly(A) mRNA for specific initiation of cDNA synthesis. Moreover, the addition of two nucleotides to the oligo(dT) segment of the primer molecule increases the specificity of the priming event in a nonhomogeneous population of mRNA. The oligodeoxynucleotide primer d(pT_8-G-C) which was determined to be complementary to the poly(A) junction of bovine GH mRNA was used to analyze the 3′-terminal noncoding sequence of the mRNA from an enriched fraction of bovine anterior pituitary mRNA.

Reverse transcriptase directed cDNA synthesis to enriched bovine GH mRNA was utilized in two separate approaches

to sequence the 3′-terminal region of the mRNA. The dideoxy chain terminators were used with the specific d(pT_8-N-N′) primer of bovine GH cDNA synthesis to initially determine the 3′-terminal sequence of the mRNA. Subsequently, the Maxam & Gilbert (1977) method for DNA sequencing was used to analyze bovine GH cDNA specifically initiated with [5′-^{32}P]d(pT_8-G-C). In this manner, the complete 3′-nontranslated sequence of bovine GH mRNA was obtained.

The successful application of the d(pT_8-N-N′) primer selection technique to bovine GH mRNA (Figure 2) and the subsequent sequence determination of the 3′-noncoding region (Figure 3) demonstrate this method's potential for sequence analysis of any enriched fraction of poly(A) mRNA. Although primers with the sequence d(pT_8-N) may be sufficient to specifically initiate cDNA synthesis with highly purified mRNA templates, such as viral mRNAs (Zimmern & Kaesberg, 1978; McGeoch & Turnbull, 1978), the more specific primers with two nucleotides following the oligo(dT) segment offer greater selectivity for specific initiation of cDNA synthesis in a nonhomogeneous population of mRNAs. Our preparation of bovine GH mRNA was estimated to be approximately 70–80% pure by in vitro translation (data not shown). Screening of the 12 oligodeoxynucleotide sequences strongly indicated that d(pT_8-G-C) functioned as a specific primer of bovine GH cDNA synthesis (Figure 2). A small amount of cDNA was initiated by the primer d(pT_8-G-T) which produced a much less intense banding pattern identical in character with the fragments generated with the sequence d(pT_8-G-C). However, the fragments terminated in the presence of the d(pT_8-G-T) primer were obscured by a faint ladder of T residues. It is likely that the T ladder effect indicates some nonspecific initiation on the poly(A) tail of the mRNA and may indicate an impurity in the d(pT_8-G-T) preparation. The presence of a small amount of contaminating d(pT_8-G) would be sufficient to initiate cDNA synthesis on the bovine GH mRNA template and produce the observed termination fragments, while the presence of d(pT_8) would produce the ladder of T termination fragments by initiating cDNA synthesis nonspecifically on the poly(A) tail of the mRNA. Additionally, there is the possibility that the sequence d(pT_8-G-T) may still be able to serve as a primer of cDNA synthesis with a single mismatched base pair to the GH mRNA template.

Although we have not critically examined the lower limits of mRNA enrichment required for detection of specific initiation of cDNA synthesis, it is clear that a homogeneous mRNA template is not essential for application of this technique. In recent experiments a mRNA preparation containing only 50% GH mRNA sequences, as estimated by in vitro translation, proved to be adequate for sequence analysis by this method (N.L.S., unpublished experiments). The selection of the specific complementary primer does, however, require that the contaminating mRNA sequences do not interfere with the desired mRNA sequence and that initiation of cDNA synthesis occurs at only one site on the specific mRNA template. With these limitations taken into consideration, the most intense banding pattern on the primer screening gel (Figure 1) should indicate the appropriate complementary primer sequence for the enriched species of mRNA.

The phased priming of cDNA synthesis for sequence analysis as described here cannot lead to the complete 3′-terminal noncoding sequence of a particular mRNA in every instance, depending on the length of this region. However, the technique may prove valuable in other respects. Double-stranded cDNA cloning methods sometimes fail to generate complete copies of the mRNA sequence due to incomplete second strand synthesis. Thus, the cloned sequences are lacking the 3′-terminal portion of the mRNA sequence. Sequence analysis of such clones in combination with the use of oligodeoxynucleotide primers to determine the poly(A)-adjacent sequence of the mRNA, purified by selective binding to the cloned sequence, may provide the necessary overlap to analyze the entire mRNA sequence. Furthermore, identification of specific primers for cDNA synthesis may be utilized to generate unique cDNA probes for screening recombinant DNA clones. In some instances screening is hampered by the purity of the cDNA probe. The use of a specific d(pT_8-N-N′) primer for cDNA synthesis would facilitate the synthesis of a pure probe from a heterogeneous population of mRNAs. Finally, recent studies focused on the 5′ terminus of ovalbumin mRNA have shown heterogeneity in the nucleotides immediately adjacent to the cap structures (Malek et al., 1979). The analysis of 3′-terminal mRNA sequences by phased priming at the poly(A) junction would facilitate the direct examination of this region for possible heterogeneity in the sequence adjoining the poly(A) segment.

The 3′-terminal noncoding sequence of bovine GH mRNA contains several interesting features (Figure 4). First, the sequence is rich in C residues. Of the 104 bases in this nontranslated region, 34 are C residues. Also, many stretches of C residues are present, the longest one of which occurs at nucleotides 71–80 where 9 C residues are interrupted by only one U residue. The significance of such regions in unknown. However, the GH mRNAs from rat (Seeburg et al., 1977) and human (Martial et al., 1979) also contain many repeats of C residues. Second, the hexanucleotide AAUAAA generally found in the 3′-noncoding region of eucaryotic mRNAs is also present in bovine GH mRNA. This sequence occurs approximately 20 nucleotides from the poly(A) tail of mRNAs (Proudfoot & Brownlee, 1976). In bovine GH mRNA, the hexanucleotide is located at nucleotides 19–24. The significance of this sequence is still unknown, but it appears to be an essential part of the 3′-noncoding sequence of many eucaryotic mRNAs. Third, the sequence for bovine GH mRNA strongly resembles that for rat and human GH mRNAs in several respects. The length of the 3′-terminal noncoding sequences for rat and human GH mRNAs is 105 and 108 bases, respectively. Our sequence analysis of bovine GH mRNA shows the 3′-terminal noncoding region to be 104 nucleotides in length. Thus, it appears that there has been a conservative evolutionary pressure on the length of the GH mRNA 3′-terminal noncoding region. In comparison, the length of this segment in β-globin mRNA from rabbit is 38 and 39 nucleotides shorter than that from the mouse and human species, respectively (Proudfoot, 1977; Konkel et al., 1978). Additionally, the bovine GH mRNA displays a maximum of 50% and 82% sequence homology with rat and human GH mRNAs, respectively. Martial et al. (1979) have reported 38% homology between the rat and human species with a maximum overlap of 55%. It is interesting to note in this respect that the terminal trinucleotide sequence, AUC, which is adjacent to the poly(A) tail in both the rat and human mRNAs is displaced from the poly(A) junction in the bovine GH mRNA sequence by the two nucleotides GC. Furthermore, there is extensive homology surrounding the region of the hexanucleotide sequence, AAUAAA, for the GH mRNA from these three species. When a 30-nucleotide sequence of this region is aligned to maximize the base homology, 23 and 27 bases from the rat and human sequences, respectively, are identical with those of bovine GH mRNA. The homology for

the rat and human species in this same 30 base sequence is 27 nucleotides (Martial et al., 1979). There is also substantial homology in this region among the β-globin mRNAs of rabbit, mouse, and human (Proudfoot, 1977; Konkel et al., 1978). Thus, it appears that the unknown function of this region of mRNA structure exerts a conservative pressure for sequence retention in homologous mRNAs.

What role the 3′-nontranslated region of mRNA plays in the function of the molecule is still unclear. A full understanding of mRNA structure will require the comparison of many mRNA sequences. The wide variety of methods available for determination of mRNA sequences offers the potential to examine such questions. The use of oligodeoxynucleotide primers of the general structure $d(pT_8\text{-}N\text{-}N)$ to determine poly(A)-adjacent sequences in mRNAs should facilitate these comparative studies.

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