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Construction and characterization of a cDNA clone containing a portion of the bovine prolactin sequence

John H.Nilson[+], Arlen R.Thomason[*], Sarah Horowitz[+], Nancy L.Sasavage[+], John Blenis[+], Robert Albers[+†], Winston Salser[*] and Fritz M.Rottman[+]

[+]Department of Biochemistry, Michigan State University, East Lansing, MI 48824, and [*]Department of Biology, University of California, Los Angeles, CA 90024, USA

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ABSTRACT

Poly(A)-containing RNA from the bovine anterior pituitary has been used as a template for the enzymatic synthesis of double-stranded cDNA. The resulting double-stranded cDNA was inserted into the Pst I site of pBR322 with the oligo(dG)-oligo(dC) tailing technique and subsequently cloned in *E. coli* χ1776. Clones containing sequences complementary to prolactin mRNA were identified by colony hybridization with partially purified prolactin cDNA. A 250 base pair sequence from one prolactin positive clone was extensively characterized and shown to contain the coding information for amino acids 119-192 of authentic bovine prolactin. The recombinant DNA from this clone was covalently attached to diazotized aminocellulose and used to purify prolactin mRNA from a mixture of mRNAs.

INTRODUCTION

Control of gene expression in eucaryotes can occur at several levels including transcription, post-transcriptional processing events, and translation. A central question regarding selective gene expression involves the extent to which these control levels overlap, and whether the degree of overlap is unique for different classes of mRNA.

One approach to this problem is to study and elucidate the regulatory events associated with the biosynthesis of mRNAs coding for polypeptide hormones. In the anterior pituitary gland, or in pituitary tumor cells, increases in prolactin and growth hormone levels coincide with increases in their respective cytoplasmic mRNA levels (1-10). These changes suggest that some alteration in the biosynthesis of the mRNAs coding for prolactin and growth hormone represent the rate-limiting or regulatory step in the synthesis of these two pituitary hormones. However, it is still not known whether the changes in prolactin or growth hormone mRNA levels reflect primarily a transcriptional or post-transcriptional event.

In order to determine whether prolactin or growth hormone mRNA biosynthesis is regulated at a transcriptional or post-transcriptional level,

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homogeneous DNA affinity probes complementary to each mRNA are required to evaluate potential differences in the synthesis, processing and stability of these mRNAs. Recently, Harpold et al. (11) reported on the cloning of cDNA sequences complementary to rat growth hormone (GH) mRNA, while Gubbins et al. (12) have constructed a cDNA clone complementary to rat prolactin (PRL) mRNA. In this study, we report on the construction and characterization of a cDNA clone that contains part of the gene sequence which codes for bovine prolactin. In addition, we show that the cloned prolactin DNA sequence can be used as an affinity probe for the isolation of bovine PRL mRNA.

MATERIALS AND METHODS

Materials. All radioactive nucleoside triphosphates were obtained from Amersham. Terminal transferase was prepared in the laboratory of R.L. Ratliff by J. Isaacson, C. Manske and W. Salser. Proteinase K was obtained from E.M. Labs, T_4-polynucleotide kinase from New England Biolabs, S_1-nuclease from Miles, bacterial alkaline phosphatase from Worthington, and lysozyme from Sigma. Reverse transcriptase was a generous gift of Dr. Joseph Beard (Life Sciences Inc., St. Petersburg, FL). All restriction enzymes were purchased from Bethesda Research Laboratories. The digestions with restriction enzymes were carried out using the conditions suggested by the supplier for each enzyme.

Isolation of Bovine Pituitary mRNA. Polysomes were prepared from fresh bovine anterior pituitaries by a modification of the magnesium precipitation method as described by Palmiter (13). Modifications included the use of 0.2% instead of 2.0% Triton X-100, and a brief proteinase K treatment (250 µg/ml, 37°C, 30 minutes in buffer containing 1.0% SDS) of the isolated polysomes prior to phenol-chloroform extraction. Poly(A)-containing RNA was isolated from total polysomal RNA by oligo(dT)-cellulose chromatography as described previously (14).

Prolactin mRNA was purified from the poly(A)-containing RNA by centrifugation on 5-20% (w/w) sucrose gradients in 10 mM Tris (pH 7.5), 1 mM EDTA, and 0.2% SDS. Centrifugation was performed at 35,000 rpm for 12 hours at 22°C in a Beckman SW-41 rotor. The gradients were fractionated and the absorbance at 260 nm determined with a Gilford 240 recording spectrophotometer. The RNA from each fraction was recovered by ethanol precipitation and translated in the wheat germ cell-free system as previously described (15). Fractions containing PRL mRNA were pooled and centrifuged a second time as described above to further purify the PRL mRNA.

Synthesis of Single-stranded cDNA. The synthesis of cDNA to poly(A)-containing RNA was carried out essentially as described by Myers et al. (16). The components of the reaction were: 50 mM Tris (pH 8.3), 37.5 mM KCl, 8 mM $MgCl_2$, 250 µM each of dATP, dGTP, dTTP, [^{3}H]-dCTP (0.08 Ci/mMole), 400 µM dithiothreitol, 4 mM sodium pyrophosphate, 5 µg/ml oligo(dT)$_{12-18}$, 100 µg/ml poly(A)-containing RNA, and 300 units/ml reverse transcriptase. The 500 µl reaction was incubated at 37°C for one hour, extracted with chloroform-isoamyl alcohol (24:1), and then chromatographed over Sephadex G-50 in 20 mM Tris (pH 7.5), 2 mM EDTA, 100 mM NaCl, and 0.2% SDS. The fractions containing cDNA were pooled and ethanol precipitated. The RNA was hydrolyzed from the cDNA by incubation in 0.6 N NaOH, 0.01 M EDTA for two hours at 37°C. The mixture was neutralized with 6 N HCl and the cDNA precipitated by adding $MgCl_2$ to 0.01 M, 18S rRNA to 10 µg/ml, and two volumes of ethanol (-80°C, two hours). The final cDNA pellet was dissolved in water and stored at -20°C.

When cDNA was synthesized from PRL mRNA for use in colony hybridization, the RNA concentration was lowered to 20 µg/ml, and 10 µM [α-^{32}P]-dCTP (200 Ci/mMole) was used in place of [^{3}H]-dCTP.

Synthesis of Double-stranded cDNA. The reaction conditions for synthesis of the second cDNA strand were: 50 mM Tris (pH 8.3), 37.5 mM KCl, 8 mM $MgCl_2$, 250 µM each of dATP, dGTP, and dTTP, 100 µM [^{3}H]-dCTP (2.5 Ci/mMole), 10 µg/ml cDNA, and 400 units/ml of reverse transcriptase. The reaction was incubated at 37°C for two hours, extracted with chloroform-isoamyl alcohol and chromatographed on Sephadex G-50 as described above. The fractions containing the cDNA were pooled, adjusted to 40 µg/ml with yeast tRNA and ethanol precipitated. The 3'-terminal hairpin loop was opened by digesting the cDNA with S_1-nuclease at 37°C for one hour. The components of this reaction were: 0.3 M NaCl, 0.05 M potassium acetate (pH 4.5), 1 mM $ZnSO_4$, 20 µg/ml cDNA, and 100 units S_1-nuclease per µg of total nucleic acid. In order to terminate the S_1 digestion, EDTA and SDS were added to 20 mM and 0.2%, respectively. The reaction mixture was then extracted with chloroform-isoamyl alcohol, and the cDNA was ethanol precipitated.

Construction of Hybrid Plasmids. Purified pBR322 DNA (17,18) was restricted with Pst I, phenol extracted, ethanol precipitated and dissolved in H_2O. The addition of dGTP to the pBR322 DNA (adjusted to 90 µg/ml) was performed in a reaction mixture containing 200 mM Hepes (pH 6.9), 0.5 mM β-mercaptoethanol, 50 µM [^{3}H]-dGTP (10 Ci/mMole), 1 mM $CoCl_2$ and 480 units/ml of terminal transferase for 15 minutes at 37°C. These conditions

resulted in the addition of approximately 10 nucleotides to each end of the plasmid. The reaction was stopped by adding EDTA to 10 mM and the mixture was stored at -20°C. No further purification of the tailed pBR322 DNA was performed before its use in the annealing reaction (12).

The double-stranded cDNA was tailed with [α-^{32}P]-dCTP (20 Ci/mMole) using the same conditions described above except that the concentration of double-stranded cDNA was 10 μg/ml and the terminal transferase concentration was 240 units/ml. The length of the oligo(dC) tails was approximately 17 nucleotides. The reaction was stopped and the mixture stored as described above.

The oligo(dG)-tailed pBR322 DNA and the oligo(dC)-tailed double-stranded cDNA were diluted to 0.7 nM each (1.82 μg/ml and 0.3 μg/ml, respectively) in 100 mM NaCl, 10 mM Tris (pH 8.3), and 10 mM EDTA. The DNAs were annealed at 51°C for 30 minutes and then cooled slowly to room temperature overnight (19). The extent of annealing was monitored by both electron microscopy and electrophoresis in a 1% agarose gel (data not shown).

Transformation. The transformation procedure used in this study was according to Villa-Komaroff et al. (20), except for the following modifications. The L-broth (21) was supplemented with 100 μg/ml diaminopimelic acid (DAP) and 20 μg/ml thymidine (THY). E. coli χ1776 cells were grown to a density of A_{600} = 0.3. The transformation mixtures were composed of 0.2 ml cells and 0.05 ml recombinant plasmid (1.8 μg/ml). Three ml of brain heart infusion (BHI) soft agar (0.7%) was added to each aliquot of the transformation mixture before plating on BHI agar (1%) plates containing 15 μg/ml tetracycline (TET).

Screening of Recombinant Plasmids. The colony hybridization procedure used in the present study is a modification of that originally described by Grunstein and Hogness (22). Individual colonies were transferred to a millipore filter which was supported by 1.5% L-agar (containing DAP, THY and TET), and incubated overnight at 37°C. The bacteria were then lysed and their DNA fixed to the nitrocellulose filters as described by Gubbins et al. (12). The DNA-containing filters were pre-hybridized in a silanized glass petri dish for one hour at 43°C in 50% formamide containing 5X SSCP (23), 0.5% SDS and 100 μg/ml sheared and denatured salmon sperm DNA. [^{32}P]-PRL cDNA was then added (10^6 cpm/filter) and the hybridization was continued for at least 15 hours at 43°C. The filters were washed three times with 50% formamide containing 5X SSCP and 0.5% SDS and then two times with 2X SSCP as described by Benton and Davis (23). Positive colonies were visualized by

exposing the blotted filters overnight at -80°C to Kodak RP-X-OMAT film with DuPont Chronex lightning plus intensifying screen.

Plasmid Amplification and Isolation. Each clone was rescreened by colony hybridization and then grown overnight in L-broth supplemented with DAP (100 μg/ml), THY (20 μg/ml) and TET (15 μg/ml). An aliquot of the overnight culture was then diluted 1:33 into one liter of L-broth + DAP + THY. When the cells had grown to a density corresponding to A_{600} = 0.7-0.8, chloramphenicol (15 mg/ml in 100% ethanol) was added to a final concentration of 75 μg/ml, and the incubation continued for 15-20 hours. The cells were then pelleted, washed and frozen in dry ice-ethanol.

A slight modification of the procedures described by Godson and Sinsheimer (24) and Clewell and Helinski (25) was used to lyse the cells with neutral detergent and to prepare cleared lysates. Frozen cell pellets from a one-liter culture were thawed and suspended in 10 ml of cold 25% sucrose, 0.05 M Tris (pH 8.0). Two ml of fresh, cold lysozyme (10 mg/ml) in 25% sucrose, 0.05 M Tris (pH 8.0) was added to the cells. The mixture was swirled intermittently on ice for 10 minutes. Then two ml of cold, 0.5 M EDTA was added, and the mixtures were again swirled intermittently on ice for five minutes. A total of 15 ml of NP-40 solution (0.2% NP-40, 6.25 mM EDTA, 50 mM Tris, pH 8.0) was added with mixing, and the tubes were left for 10 minutes at room temperature. The lysates were then centrifuged at 23,500 xg for 35 minutes at 4°C. The supernatants were treated with proteinase K (100 μg/ml) and SDS (0.1%) for 30 minutes at 37°C, diluted 1:1 with water, extracted with phenol-chloroform, and ethanol precipitated.

Recombinant plasmid DNA was further purified from RNA and *E. coli* chromosomal DNA by centrifugation in preparative CsCl bouyant density gradients essentially as described by Clewell and Helinski (25). Approximately 150 μg of plasmid DNA were routinely obtained from one liter of *E. coli* $_{\chi}$1776.

The isolation, growth, and lysis of recombinant bacteria were carried out in a P2 containment laboratory as specified by the NIH guidelines for recombinant DNA research.

DNA Sequence Analysis. The chemical modification and cleavage reactions for DNA described by Maxam and Gilbert were used to sequence the Hae III and Hha I restriction fragments of the cloned DNA (26). Thin sequencing gels described by Sanger and Coulson (27) were employed for all analyses.

RESULTS

Characterization of Bovine Pituitary mRNA and cDNA. The absorbance profile of bovine pituitary poly(A)-containing RNA after sedimentation in linear 5-20% (w/w) sucrose gradients is shown in Fig. 1. The profile is characterized by a major peak which sediments at approximately 12S and a contaminating 18S rRNA peak. The 12S peak contains a slight shoulder on its leading edge. The primary cell-free translation products of mRNA isolated from fractions A and C of the material shown in Fig. 1 were identified as PRL and GH, respectively. These results are in agreement with our previous observation that in the anterior pituitary, PRL and GH mRNA represent the major components of poly(A)-containing mRNA sequences, with PRL mRNA being more abundant than GH mRNA (15).

We chose to clone double-stranded cDNA synthesized from pituitary poly(A)-containing RNA since the majority of cDNA sequences synthesized from this RNA fraction could be expected to be complementary to PRL mRNA. How-

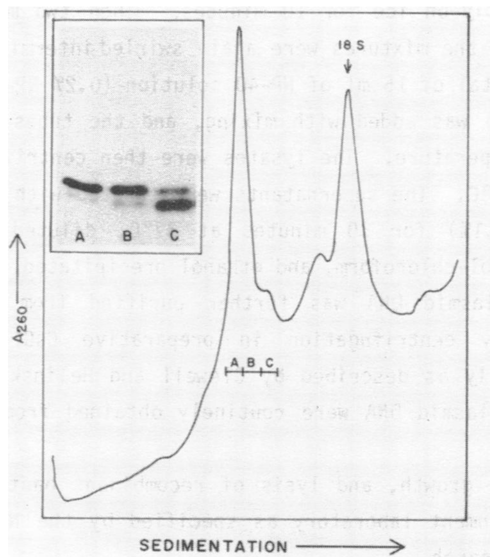

Figure 1. Sucrose gradient centrifugation of poly(A)-containing RNA from the bovine anterior pituitary. Poly(A)-containing RNA was centrifuged in 5-20% (w/w) sucrose gradients as described in MATERIALS and METHODS. The areas A, B, and C indicate the fractions of RNA that were recovered from the gradient and translated in the wheat germ cell-free system. The cell-free translation products were electrophoresed in a SDS-polyacrylamide slab gel and then fluorographed. The fluorograph is shown in the inset. The top band is pre-prolactin while the lower band is pre-growth hormone.

ever, in order to identify clones containing PRL sequences, it was necessary to prepare a hybridization probe specific for PRL mRNA. This was accomplished by synthesizing cDNA to PRL mRNA which was isolated from the central portion of the 12S peak (fraction A, Fig. 1) after two sucrose gradient sedimentation steps. It is important to note that the PRL mRNA prepared in this manner contained little or no GH mRNA sequences when assayed by cell-free translation in the wheat germ system (cf. A, inset Fig. 1).

Construction and Detection of cDNA Clones Complementary to PRL mRNA. Double-stranded cDNA to poly(A)-containing mRNA was synthesized by using reverse transcriptase for both the first and second strand (12). As indicated in Track A of Fig. 2, the length of the first strand was approximately 880 nucleotides. This is close to the expected size for full-length cDNA synthesized from a PRL mRNA template (28). In contrast, the length of the denatured double-stranded cDNA (containing the 3'-terminal hairpin loop) was approximately 1470 nucleotides, about 80% of its expected full length (Fig.

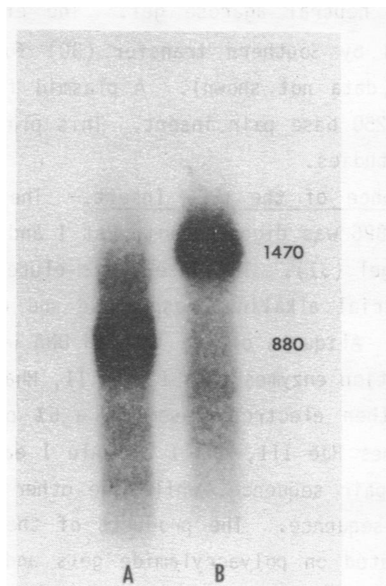

Figure 2. Alkaline-agarose gel electrophoresis of PRL cDNA. Electrophoresis was performed in a horizontal 1.4% alkaline agarose gel (0.03 M NaOH, 0.002 M EDTA). In order to size the single and double-stranded cDNAs (lanes A and B, respectively), Hae III restriction fragments of ϕX174 DNA were electrophoresed in parallel. The gel was dried and exposed to Kodak RP-X-OMAT film with Dupont Chronex lightning plus intensifying screen at -80°C. The adjacent numbers indicate the size, in nucleotides, of the single and double-stranded PRL cDNAs.

2, Track B). The same results were obtained when the Klenow fragment of _E. coli_ DNA polymerase I was used to synthesize the second strand (data not shown).

The double-stranded cDNA was inserted into the Pst I site of pBR322 using the oligo(dG)-oligo(dC) joining procedure (20). This approach has been shown to result in the reconstruction of a Pst I site at each end of the double-stranded cDNA insert (29). The hybrid plasmid was then used to transform _E. coli_ χ1776 as described earlier.

A modification of the _in situ_ hybridization technique described by Grunstein and Hogness (22) was used to screen tetracycline resistant bacteria for PRL-containing sequences. The radioactive probe was [^{32}P]-cDNA synthesized from purified PRL mRNA (MATERIALS and METHODS and Fig. 1). The colonies showing the highest degree of hybridization were regrown in liquid culture and their recombinant plasmids amplified with chloramphenicol. The isolated plasmid DNAs were treated with Pst I and electrophoresed in a neutral agarose gel. The electrophoresed DNAs were further characterized by Southern transfer (30) followed by hybridization with [^{32}P]-PRL cDNA (data not shown). A plasmid from one of the positive colonies contained a 250 base pair insert. This plasmid was designated pBP6 and used in further studies.

Nucleotide Sequence of the pBP6 Insert. The DNA isolated from the recombinant plasmid pBP6 was digested with Pst I and then electrophoresed in a 6% polyacrylamide gel (31). The insert was eluted from the gel, dephosphorylated with bacterial alkaline phosphatase and end-labeled with polynucleotide kinase (26). Aliquots of the labeled DNA were digested with one of the following restriction enzymes: Alu I, Hpa II, Hha I, Bgl II, Hae III, Taq I, or Hinc II, and then electrophoresed in a 6% polyacrylamide gel. The restriction enzymes Hae III, Hha I and Alu I each resulted in a single cut in the 250 base pair sequence, while the other restriction enzymes did not cut within this sequence. The products of the Hae III and the Hha I digestion were separated on polyacrylamide gels and sequenced according to Maxam and Gilbert (26). Figure 3 summarizes the nucleotide sequence of the insert and the amino acids predicted from one potential reading frame of this sequence. The correlation between the known amino acid sequence of bovine prolactin (32) and the amino acid sequence deduced from the inserted sequence of pBP6 permits a positive identification of this clone as one containing a portion of the sequence for PRL.

Construction of a PRL-DNA Affinity Support. DNA from pBP6 was linear-

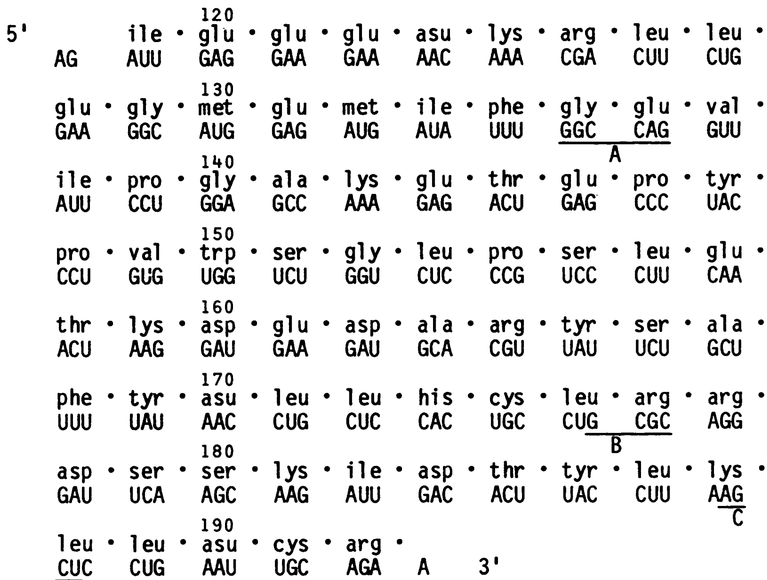

Figure 3. Nucleotide sequence dervied from the insert of pBP6 and the corresponding amino acid sequence of bovine prolactin. The sequences underlined in A, B and C are recognition sites for Hae III, Hha I, and Alu I, respectively. The total length of the insert, including the tails, is 250 base pairs.

ized by digestion with Eco RI and covalently coupled to diazotized amino-cellulose as described by Childs et al. (33). In order to determine the binding specificity of the pBP6 cellulose, a mixture of mRNAs containing sequences specific for PRL, GH and ovalbumin was incubated under hybridization conditions with the cellulose-bound DNA. Cell-free translation in the wheat germ cell-free system was then used to assess which mRNAs were selectively bound to the pBP6 cellulose. The translation products of the mRNA mixture before its hybridization to pBP6 cellulose are shown in lanes B and F of Fig. 4. The mRNAs for PRL, GH and ovalbumin were present in approximately equal amounts. The translation products of the mRNAs that did not hybridize to the pBP6 cellulose, pre-GH and ovalbumin, are shown in lane C. In lane D, the translation products of mRNAs which eluted with hybridization buffer at 22°C are shown. The eluted mRNA was principally PRL. The translation products of the mRNAs which hybridized to pBP6 cellulose and required elution with 99% formamide at 65°C are shown in lane E. In this case, PRL

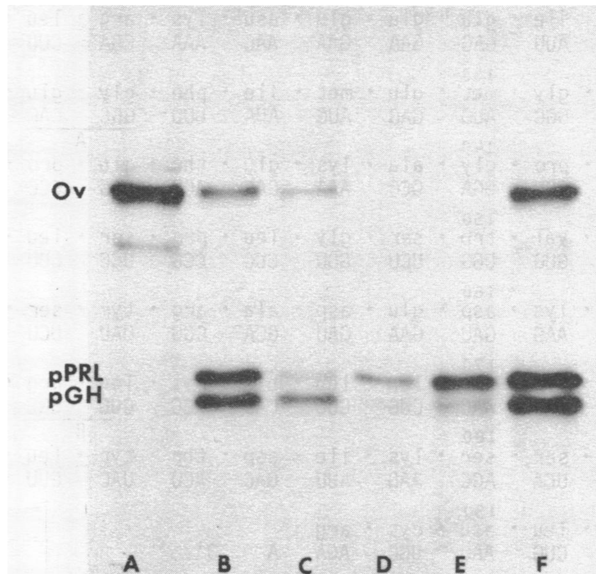

Figure 4. Fluorogram of cell-free translation products directed by mRNAs from the unhybridized and hybridized fractions of pBP6 cellulose. The condi tions used for hybridization, washing and elution of the mRNAs from the pBP6 cellulose were those previously described (33). The translation products are from: lane A, ovalbumin mRNA standard; lanes B and F, ovalbumin, PRL and GH mRNA before hybridization; lane C, unhybridized mRNA; lane D, mRNA eluted with the room temperature wash in hybridization buffer; lane E, mRNA eluted with 99% formamide at 65°C. Ov, ovalbumin; pPRL, pre-prolactin; pGH, pre-growth hormone.

mRNA was the most abundant component. It should be noted that the very low amounts of GH mRNA could be the result of non-specific interaction with the cellulose fines. Taken together, the results in Fig. 4 suggest that the pBP6 cellulose affinity support can be used to purify PRL mRNA from a mixture of mRNAs.

DISCUSSION

We have described the construction and characterization of a cDNA clone which contains a portion of the structural gene coding for bovine prolactin. Since bovine pituitary PRL mRNA represents as much as 60-70% of the total mRNA population (15), double-stranded cDNAs synthesized from poly(A)-containing RNA can be used without any further purification steps to efficiently

generate PRL-containing clones. Furthermore, bovine PRL mRNA can be readily purified by sucrose gradient sedimentation. [^{32}P]-cDNA synthesized from this purified mRNA can be used as a screening probe for PRL-containing clones in colony hybridization.

Generally about 2 µg of polysomal poly(A)-containing RNA can be isolated from one gram of anterior pituitary tissue. From 10 µg of poly(A)-containing RNA we synthesized 1 µg of single-stranded cDNA and subsequently, 0.2 µg of double-stranded cDNA. From this amount of double-stranded cDNA, we obtained 47 transformants, two of which hybridized strongly to the pPRL cDNA screening probe.

As a result of the oligo(dG)-oligo(dC) tailing technique which was used to construct the hybrid plasmids, the clones obtained in this study contained functional Pst I sites at the ends of their inserted sequences. The presence of two Pst I sites facilitated the excision of the inserted sequence from the plasmid for sequence analysis. In addition, the clones obtained in this study were sensitive to ampicillin. However, it should be noted that while the Pst I site of pBR322 is located in the ampicillin gene (17), cloning into this site does not always inactivate the enzymatic activity of the gene (20).

The cloned sequence in pBP6 is 250 nucleotides in length. Since the complete amino acid sequence of bovine PRL has been reported by several groups (for review see reference 32), it is possible to establish the correct reading frame from the nucleotide sequence of the cloned insert and determine whether it represents a coding sequence for the amino-terminal or carboxyl-terminal end of PRL. The data in Fig. 3 indicates that the inserted sequence contains the complete coding information for amino acids 119-192 of bovine PRL (199 total amino acids). We were also able to locate within this sequence the restriction sites for Hha I, Alu I, and Hae III. As expected, each of these restriction sites was present only once in the sequence. The amino acid sequence predicted from the cloned sequence agrees with that established by amino acid sequencing techniques, confirming that the insert is a portion of the gene coding for bovine PRL.

Parlow and Shome (34) report that the overall amino acid sequence homology between rat and bovine PRL is 45%. Gubbins et al. have recently characterized a cloned sequence that codes for rat prolactin (12). They sequenced a region of 485 base pairs corresponding to the amino acids comprising the signal peptide of rat pre-prolactin and also amino acids 1-145. The amino acid sequence homology for the corresponding segments of

bovine and rat prolactin (119-145) is 60%. In contrast, the nucleotide sequence homology for this region is 78%.

The cell-free translation data in Fig. 4 indicates that the pBP6 cellulose can be used to hybridize PRL mRNA selectively from a mixture of mRNAs. The PRL mRNA fraction that was eluted from the affinity support with 99% formamide contained a small amount of GH mRNA. It remains to be determined whether the trace of GH mRNA is the result of non-specific binding to the pBP6 cellulose.

The availability of a DNA-affinity support specific for bovine PRL mRNA will facilitate studies on rates of synthesis, processing, transport and stability of this mRNA. Since these studies will be carried out in primary cultures of bovine pituitary cells obtained from adult animals, it will be of interest to determine whether the regulation of PRL mRNA expression in the cow is similar to that operating in the rat pituitary or in rat pituitary tumor cells.

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†Present address: Department of Chemistry, Calvin College, Grand Rapids, Michigan 49506

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