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Construction of bacterial plasmids that contain the nucleotide sequence for bovine corticotropin-β-lipotropin precursor

(recombinant DNA/DNA)

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ABSTRACT mRNA that encodes the common peptide precursor for the hormones corticotropin and β-lipotropin was purified from the neurointermediate lobe of bovine pituitaries, and double-stranded cDNA species synthesized from this template were cloned in *Escherichia coli* χ1776 by inserting them into the *Pst* I endonuclease cleavage site of the pBR322 plasmid using poly(dG)·poly(dC) homopolymeric extensions. Certain of the cloned cDNA inserts contain nucleotides corresponding to the complete amino acid sequence of bovine corticotropin and a coding sequence that corresponds to at least the first portion of bovine β-lipotropin. The nucleotide sequences coding for corticotropin and β-lipotropin are separated on the cDNA by a 6-base-pair sequence encoding lysine and arginine, indicating that the carboxyl terminus of corticotropin is connected on the precursor peptide with the amino terminus of β-lipotropin by these two amino acids. In addition, the cloned cDNA insert is characterized by an unusually high C+G nucleotide base content as well as by a number of DNA sequence duplications.

The pituitary hormones corticotropin (ACTH) and β-lipotropin (β-LPH), which consist of 39 and 91 amino acids, respectively, are formed from a large common polypeptide having a molecular weight of 28,500–41,000 (1–7). These two hormones themselves contain several small component peptides that have biological activities: α-melanotropin (α-MSH) and corticotropin-like intermediate-lobe peptide (CLIP) derived from corticotropin (8), and γ-LPH, β-melanotropin (β-MSH), endorphins, and [Met]enkephalin elaborated from β-LPH (9, 10). However, neither the precise locations of ACTH and β-LPH in the precursor molecule nor the primary structure or biological function of its remaining portion have been determined.

Nakanishi *et al.* (11) and Nakamura *et al.* (12) have demonstrated that glucocorticoids depress the intracellular level of the mRNA coding for the precursor. The effects of various steriods on the level of precursor mRNA correlate well with the binding specificity of the glucocorticoid receptor, suggesting that this receptor plays an essential role in regulating expression of the ACTH-β-LPH gene. Thus, the ACTH-β-LPH coding sequence provides an attractive system for studying the structure and regulation of hormonally regulated eukaryotic genes.

This report describes the construction and preliminary analysis of bacterial plasmids that carry a cDNA sequence transcribed from bovine ACTH-β-LPH mRNA.

MATERIALS AND METHODS

Synthesis of cDNA and Addition of Homopolymeric Tails. ACTH-β-LPH mRNA was purified from the neurointermediate lobes of bovine pituitaries as described (13); its molecular weight was estimated from its electrophoretic mobility to be approximately 450,000, which corresponds to 1360 nucleotides. Single-stranded cDNA was synthesized as described (14) using precursor mRNA template and avian myeloblastosis virus reverse transcriptase. The product was treated with NaOH to remove the mRNA template (15) and the second DNA strand was synthesized (16) by using avian myeloblastosis virus reverse transcriptase. The double-stranded cDNA product was treated with *Aspergillus oryzae* S1 nuclease (17) and *Escherichia coli* DNA polymerase I (15) and then was fractionated by agarose gel electrophoresis (17, 18). cDNA located in gels by autoradiography was extracted by electrophoretic elution (19) or by maceration of frozen gels (20).

Homopolymeric poly(dC) or poly(dG) tails were added, respectively, to the fractionated double-stranded cDNA and to *Pst* I-cleaved pBR322 plasmid DNA, respectively, by terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Bethesda, MD) (21, 22, §). Under the conditions used, terminal transferase added approximately 15–30 pmol of dCMP or dGMP per pmol of end double-stranded cDNA or vector DNA.

Construction and Analysis of Chimeric Plasmids. Annealing of poly(dG)-terminated pBR322 plasmid DNA (0.5 μg/ml) to 5.5–7.5 ng of poly(dC)-terminated synthetic DNA was carried out as described (21, §) at a molar ratio of vector to synthetic DNA of approximately 1.3. Under the conditions used, approximately 20% of the linear form of pBR322 DNA was converted to the circular form as monitored by electron microscopy (23) and gel electrophoresis.

E. coli χ1776 (24) was transformed by chimeric plasmids by using a modification (21) of a previously described transfection procedure (25). Tetracycline-resistant transformants were tested for sensitivity to ampicillin (25 μg/ml). Bacterial colonies containing ACTH-β-LPH DNA were identified by using an *in situ* colony hybridization procedure (21, 26). The ^{32}P-labeled mRNA used as a probe was labeled with [γ-^{32}P]ATP (700 Ci/mmol) and polynucleotide kinase (27).

Plasmid DNA in bacterial colonies that showed a positive reaction by *in situ* hybridization with ACTH-β-LPH mRNA

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Abbreviations: ACTH, corticotropin; LPH, lipotropin; MSH, melanotropin; bp, base pairs.

§ W. Rowekamp and R. A. Firtel, personal communication; G. M. Wahl, R. A. Padgett, and G. R. Stark, personal communication.

was amplified by the addition of chloramphenicol (120 μg/ml) to cultures, isolated as described (28), and dialyzed. Digestion with other restriction enzymes was carried out under conditions described by the vendors. DNA sequence analysis was performed as described by Maxam and Gilbert (29).

Containment. Work with recombinant plasmids was carried out under the P3 level of physical containment and EK2 level of biological containment as specified in the National Institutes of Health Guidelines on Recombinant DNA Research (June 1976).

RESULTS

Construction of Plasmids Carrying ACTH-β-LPH cDNA. Purified mRNA containing the precursor sequences from the neurointermediate lobe of bovine pituitaries was used in the preparation of single-stranded and double-stranded cDNA, and the DNA preparations were analyzed by electrophoresis on alkaline agarose gels under denaturing conditions; typical results are presented in Fig. 1*A*. ^{32}P-Labeled single-stranded cDNA appeared as discrete size classes (lane 1), the largest of which had a length of about 1200 base pairs (bp), which approximates the size of ACTH-β-LPH mRNA (12).

Attempts to optimize experimental conditions to increase the fraction of large single-stranded cDNA species [e.g., alteration of incubation time or temperature; modification of concentrations of KCl, oligo(dT)$_{12-18}$, deoxynucleotide triphosphates, or reverse transcriptase] had little effect on the size distribution of the ACTH-β-LPH precursor single-stranded cDNA product, although the optimal conditions used in the present study did yield a high proportion of full-length ovalbumin cDNA (unpublished data). We used total single-stranded cDNA, freed from mRNA by alkaline treatment, as a template for the second reverse transcriptase reaction.

The product of the second reverse transcriptase reaction contained a larger fraction of high molecular weight material than did the single-stranded preparation (lane 2). Electrophoresis of the S1 nuclease-treated product under denaturing conditions (lane 3) showed that it contained several discrete bands, including one approximately 1200 bp in length. The ^{32}P-labeled second strand of the double-stranded cDNA product showed an electrophoretic pattern similar to that seen for the single-stranded cDNA, suggesting that the various single-stranded cDNA species were converted to corresponding lengths of double-stranded cDNA in the second reverse transcriptase reaction.

Single-stranded cDNA, double-stranded cDNA, and S1 nuclease-treated double-stranded cDNA were analyzed also by electrophoresis on neutral agarose gels (Fig. 1*B*). The single-stranded cDNA of different size classes (lane 1) migrated faster than the corresponding double-stranded cDNA species (lane 2). Treatment of double-stranded cDNA with S1 nuclease did not affect its migration in gels (lane 3), indicating that the material was converted to an S1-resistant form and confirming that the product was, in fact, double-stranded.

Isolation of Bacterial Clones Carrying the ACTH-β-LPH DNA Sequence. In order to maximize the likelihood of obtaining clones that carry double-stranded cDNA corresponding to the full-size ACTH-β-LPH precursor message, S1 nuclease-treated total double-stranded cDNA was fractionated by neutral agarose gel electrophoresis, and zones of the gel containing double-stranded cDNA species estimated to have 950–1300 or 750–950 bp were excised and eluted. Hybrid DNA molecules were constructed by annealing *Pst* I-cleaved dG-tailed pBR322 (31) vector DNA to dC-tailed sized double-stranded cDNA species and were introduced into *E. coli* strain χ1776 by transformation.

Tetracycline-resistant transformant colonies were selected and tested by an *in situ* colony hybridization method (28) for the presence of DNA complementary to ACTH-β-LPH mRNA (Fig. 2). Fourteen clones that were positive by this procedure were identified; all were found to be sensitive to ampicillin, consistent with the presumed integration of a cDNA fragment at the *Pst* I site located within the β-lactamase gene of the pBR322 vector plasmid. Plasmid DNA obtained from these clones was purified for further study. Because use of the poly(dG)·poly(dC) joining procedure results in regeneration of *Pst* I cleavage sites at both ends of the insert (21, 32), the length of the cDNA can be determined readily by gel analysis of *Pst* I-cleaved chimeric plasmid DNA. As shown in Table 1, such inserts varied in length from 190 to 1220 bp; the two largest inserts, which were estimated by gel electrophoresis to contain 1200 and 1220 bp, were obtained from hybrid plasmids constructed by using double-stranded cDNA isolated from the 950- to 1300-bp section of agarose gels (see above).

Table 1 shows also that one *Eco*RI cleavage site is present within 10 of the 12 inserts that are at least 535 bp in length. One of the two *Eco*RI/*Pst* I fragments identified in eight inserts of various lengths had an almost identical molecular size (about

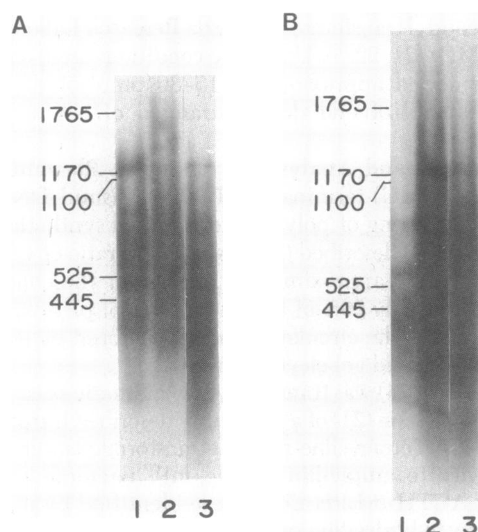

FIG. 1. Autoradiograms of ^{32}P-labeled ACTH-β-LPH DNA electrophoresed on 1.5% agarose gels. Enzymatic synthesis and labeling of single-stranded and double-stranded cDNA with [α-^{32}P]-dCTP were carried out as described (14–17). (*A*) Under denaturing conditions (30). Lanes: 1, [^{32}P]cDNA synthesized by the first reverse transcriptase reaction; 2, products of the second reverse transcriptase reaction (only the second strand was labeled with ^{32}P); 3, as lane 2 but after treatment with S1 nuclease. The *Hin*dIII cleavage products of simian virus 40 DNA were used as molecular size standards. (*B*) Under native conditions. Lanes were as in *A*.

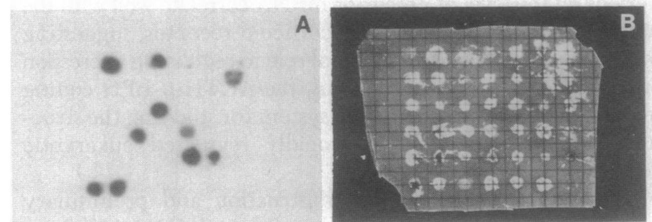

FIG. 2. Identification of bacterial colonies carrying ACTH-β-LPH DNA by *in situ* hybridization. (*A*) Autoradiogram of colonies fixed to a nitrocellulose filter and subjected to *in situ* colony-hybridization test. (*B*) Control photograph of the same nitrocellulose filter stained for DNA with ethidium bromide, indicating the retention of DNA by the filter at locations occupied by nonreacting colonies as well as those occupied by reacting colonies.

Table 1. Characterization of plasmids derived from clones identified by *in situ* colony hybridization

Plasmid	Size of inserted DNA, bp	*Eco*RI site	Size of *Eco*RI/*Pst* I fragments, bp	Orientation of cDNA insert*
pSNAC				
18	810	+	540/270	A
20	1220	+	540/680	A
21	730	−		ND
38	1200	+	1120/80	ND
26	815	+	540/275	A
31	825	+	540/285	B
34	590	+	350/240	ND
35	590	+	375/215	ND
36	860	+	460/400	ND
37	860	+	500/360	B
39	535	+	520/[15]†	B
40	310	−	—	ND
41	190	−	—	ND
42	820	+	510/310	B

The plasmids pSNAC 18, 20, 21, and 38 were constructed by using synthetic cDNA inserts having 950–1300 bp; the other plasmids included a cDNA insert that was isolated from the region of the gel occupied by DNA fragments 750–950 bp long. The synthetic DNA used for all plasmids except pSNAC 40, 41, and 42 received poly(dC) tails before size fractionation. Addition of dC tails to cDNA from the 950–1300 bp gel region after size fractionation did not yield positive colonies by the *in situ* hybridization procedure. Purified plasmid DNA was treated with either *Pst* I alone (for estimation of the size of the DNA insert) or with *Pst* I and *Eco*RI sequentially (for estimation of the size of the *Eco*RI/*Pst* I fragments). The length of DNA fragments was estimated by electrophoresis on 1.5% neutral agarose or 5% polyacrylamide gels, with *Hae* III cleavage products of CoE1 plasmid DNA as molecular weight standards (33).

* cDNA inserts in orientation A (e.g., pSNAC 20) have the ACTH coding sequence nearest the proximal end of the β-lactamase gene. The opposite orientation is orientation B. ND, not determined.

† The fragment of pSNAC 39 shown in brackets was not observed directly in the gels used for present studies, but its presence and size were inferred from other measurements. The estimated length of fragments from different fractions may be affected by the length of dGC tails on the cDNA molecule cloned.

460–540 bp) in each of the inserts. This fragment is similar in size to an *Eco*RI fragment present in the total double-stranded cDNA preparation (data not shown), suggesting that most of the cDNA inserts that we have cloned are derived from transcripts of a single species of mRNA and that the cloned inserts of various lengths correspond to sequences that start from the 3′ end of mRNA. Cleavage of the inserts of different plasmids with other restriction enzymes yielded results consistent with this interpretation.

We thus conclude that most if not all of the clones that gave a positive reaction by the *in situ* hybridization procedure contain at least part of the ACTH-β-LPH coding sequence. The presence of the entire sequence coding for ACTH, and of nucleotides comprising at least the proximal end of the β-LPH coding sequence was established directly by sequence analysis in the case of the pSNAC 20 plasmid.

Analysis of Cloned cDNA Insert in pSNAC 20 Plasmid. Computer analysis (34, 35) of the amino acid sequences of ACTH (36) and β-LPH (37) indicated that the cDNA sequence encoding glutamic acid and phenylalanine, which are situated near the carboxyl terminus of ACTH, represents the only possible locus within these two proteins for an *Eco*RI cleavage site. We therefore expected that the *Eco*RI site we had identified within the cDNA insert would be strategically located; nucleotide sequence analysis of the DNA region in the vicinity of this restriction site was performed by labeling the *Eco*RI-generated termini with [γ-^{32}P]ATP and subsequently cleaving the end-labeled fragments with *Pst* I. The two resulting fragments (fragment 1, consisting of 540 bp, and fragment 2, consisting of 680 bp) were isolated by polyacrylamide gel electrophoresis, and their nucleotide sequence was determined.

The nucleotide sequence that extends in both directions from the *Eco*RI cleavage site located within the cDNA insert corresponds to the amino acid sequences determining ACTH and β-LPH (Fig. 3). The 5′ end-labeled strand of fragment 1 shows a proximal nucleotide sequence corresponding to the two amino acids located at the carboxyl terminus of ACTH (thus verifying the location assigned by computer analysis for the *Eco*RI cleavage site), followed closely by the amino-terminal sequence of β-LPH. A short sequence of nucleotides encoding the amino acids lysine and arginine separates the coding sequences for the two peptide hormones.

The complement of the 5′ end-labeled strand of fragment 2 determines an amino acid sequence that corresponds to the remainder of the ACTH peptide. We conclude from these results that the complete coding sequence for ACTH is contained on the pSNAC 20 plasmid and, furthermore, that the carboxyl terminus of ACTH and the amino terminus of β-LPH are connected on the common precursor peptide by the sequence Lys-Arg. Because only the first 24 amino acids of ACTH are required for full biological activity of the hormone (38, 39) and because bovine and human ACTH are identical in amino acid sequence throughout this segment of the peptide (40, 41), we conclude that the pSNAC 20 plasmid carries a nucleotide sequence that encodes a peptide capable of functioning as human ACTH. Substitution of a dC for the dG at DNA position 97 of the coding strand for bovine ACTH (see Fig. 3) would substitute glutamic acid for glutamine in the peptide encoded by the cDNA insert, making the peptide identical to intact human ACTH.

Analysis of the sequenced segment of the cDNA inserted into the pSNAC 20 plasmid indicates that this segment is highly rich in G+C base pairs (68%). Cesium chloride equilibrium gradient centrifugation of *Pst* I-cleaved pSNAC 20 DNA suggests that the remainder of the insert is also rich in G+C nucleotides, because the average buoyant density of the cloned cDNA segment is 1.733 g/cm^3 (data not shown), which corresponds to a G+C composition of 72%. The high G+C content of the mRNA, as inferred from the buoyant density of the double-stranded cDNA, is consistent with the previously observed high melting temperature of ACTH-β-LPH mRNA·cDNA hybrids (unpublished data).

When subjected to computer analysis (34, 35), the data shown in Fig. 3 indicate also that the coding sequences for ACTH and at least the first segment of β-LPH contain an unusually large number of DNA sequence duplications (for example, nucleotides 93–101 and 165–173; 166–177 and 187–198; 46–55 and 73–81; 48–57 and 155–163; 139–147 and 191–202).

Treatment of the pSNAC 20 plasmid DNA with certain other site-specific endonucleases, either individually or in pairs, enabled construction of the map shown in Fig. 4. The locations of endonuclease cleavage sites were determined by gel electrophoresis carried out after simultaneous or sequential digestion of either the intact plasmid or of fragments isolated from gels after prior digestions. Cleavage sites situated within the cDNA coding sequences for ACTH and β-LPH were initially correlated with specific amino acid positions by computer determination of nucleotide sequences that could serve both as the endonuclease cleavage site and as codons that specify amino acids located in the vicinity of the cleavage site. The predicted locations for sites within those segments studied by nucleotide sequence analysis were confirmed directly. Locations of the endonuclease cleavage sites were used also in orienting the

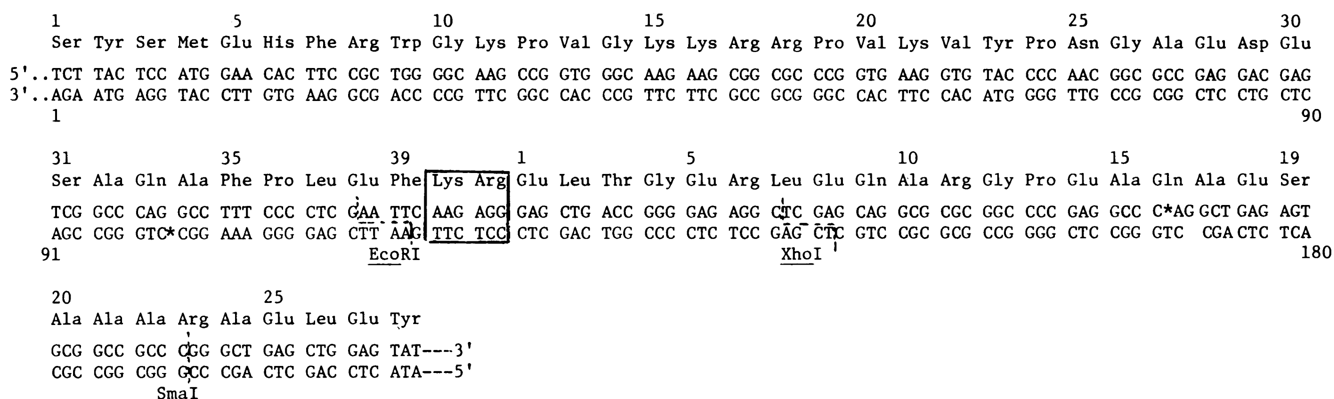

FIG. 3. Partial nucleotide sequence of the inserted ACTH-β-LPH cDNA. The sequence has been determined by 5′ end-labeling of *Eco*RI-generated fragments 1 and 2. The coding region for the two amino acids that link ACTH and β-LPH is enclosed in the box, and experimentally determined cleavage sites within the sequenced region for site-specific endonucleases are indicated. Other cleavage sites within this region, identified by computer analysis are: *Alu* I, nucleotides 125–128 and 197–200; *Hin*fI, 88–92; *Taq* I, 110–113. C* (i.e., the second C residue in the sequence C-C-T-G-G or C-C-A-G-G) was not seen on the analytical gels because this residue is methylated in *E. coli* χ1776 (24) and hence is not cleaved during the chemical modification and cleavage reactions; however, the presence of this nucleotide was inferred from the amino acid sequence. The numbers (upper numbers) before and after the boxed region indicate the amino acid residues of ACTH and β-LPH, respectively. Nucleotide positions (lower numbers) are numbered from the start of the ACTH coding sequence.

amino acid sequence of the ACTH and β-LPH with regard to the β-lactamase sequence of the pBR322 vector, as shown in Fig. 4.

DISCUSSION

Bacterial plasmids carrying cDNA transcribed from bovine ACTH-β-LPH mRNA have been shown to carry a complete ACTH coding sequence that is separated by six nucleotide base pairs from the coding sequence for β-LPH. Because the double-stranded cDNA insert present in pSNAC 20 contains about 1220 bp, it appears likely that this chimeric plasmid includes most, if not all, of the 950–1120 coding nucleotides known to be required for the bovine ACTH-β-LPH precursor (1, 2). Although the nucleotide sequence analysis reported in the present experiments extends only partially into the β-LPH coding sequence, the length of the insert and the observation that various clones containing structurally different hybrid plasmids all have a common DNA fragment that corresponds to the 3′ end of the mRNA used as template for the cDNA insert together suggest that the entire β-LPH sequence is present on the pSNAC 20 plasmid. The map distances between the various site-specific endonuclease cleavage sites shown in Fig. 4 and the *Pst* I sites that bracket the cDNA insert are consistent with this interpretation.

Although polysome run-off experiments (6) have suggested that β-LPH is located close to ACTH and somewhere near the carboxyl terminus of the precursor molecule, the structure of the peptide connecting the two hormones has not been reported. Our analysis of the nucleotide sequence of the pSNAC 20 cDNA insert in the vicinity of the *Eco*RI site at amino acids 38 and 39 of ACTH indicates that the ACTH peptide is connected to β-LPH by the sequence Lys-Arg. Because the unique *Eco*RI site in the insert has been estimated to be approximately 540 bp from the cDNA end that corresponds to the 3′ terminus of the mRNA and because this mRNA contains a poly(A) sequence approximately 70 nucleotides long at the 3′ end (unpublished data), we conclude that the carboxyl terminus of β-LPH (which consists of 91 amino acids, corresponding to 273 nucleotides) is located about 250 bp from the 3′ end of the coding region of the largest molecules of precursor mRNA.

It is noteworthy that the pair of basic amino acid residues found at the junction of bovine ACTH with β-LPH commonly connects two peptides in a precursor molecule. For example, the Lys-Arg sequence links β-MSH with β-endorphin (36), and the C peptide with the A chain of insulin (42). For separation of the ACTH and β-LPH peptide hormones, the sequence Lys-Arg must be specifically eliminated; the enzymatic mechanism responsible for this proteolytic process remains to be elucidated.

The observed high G+C content in the ACTH-β-LPH precursor coding sequence is noteworthy, because the overall G+C content of bovine DNA is only 39% (43). This observation, plus the presence of an unusual series of duplicated sequences within

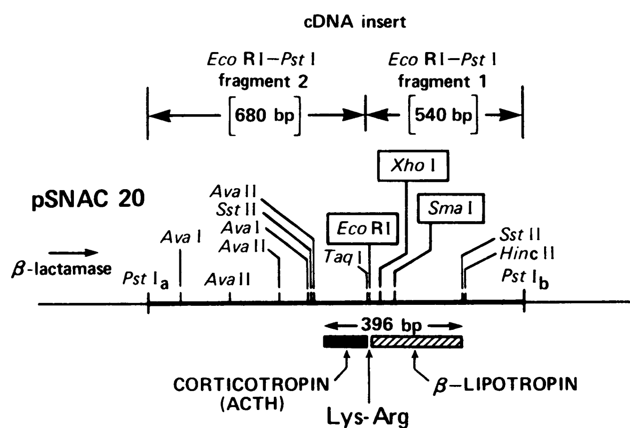

FIG. 4. Map of cDNA insert of pSNAC 20 plasmid. The bars indicate the coding sequences for bovine ACTH and β-LPH. The locations of endonuclease cleavage sites were determined from agarose gel electrophoresis patterns after simultaneous or sequential digestions of either intact plasmid DNA or fragments isolated from gels after previous digestions. The boxed cleavage sites shown above the ACTH and β-LPH genes were assigned to specific amino acid positions by direct DNA sequence analysis (see Fig. 3). The position of the Lys-Arg sequence within the 396-bp sequence that corresponds to the ACTH-β-LPH part of the precursor molecule is shown, as are the locations of *Eco*RI fragments 1 and 2 that have been end-labeled and used for DNA sequence analysis. The sizes of the two segments of the cDNA insert that are divided by the *Eco*RI cleavage site between amino acids 38 and 39 of the ACTH coding sequence have been estimated from gel electrophoresis data. The orientation of the cDNA insert in pSNAC 20, with respect to the orientation of the β-lactamase coding sequence, was determined by using known endonuclease cleavage sites on the pBR322 vector plasmid. The *Xho* I site within the β-LPH coding sequence is cleaved also by *Taq* I and *Ava* I. The *Sma* I site is cleaved also by *Ava* I. Additional cleavage sites identified by computer within the DNA segment studied by sequence analysis are listed in the legend to Fig. 3.

the segments of the insert that encode both of its major component peptides, may suggest that the coding regions for bovine ACTH and β-LPH have evolved by a series of genetic duplications. In certain instances, mismatch between two largely homologous DNA segments occurs by addition or deletion of one or two nucleotide base pairs within the segment, resulting in a shift of translational reading frame. Thus, the amino acid sequence encoded by such segments may be different, despite the extensive sequence homology observed at the nucleotide level.

Double-stranded cDNA coding sequences for mouse dihydrofolate reductase (21) and for rat proinsulin (31) recently have been expressed phenotypically or immunologically when inserted into the *Pst* I cleavage site of pBR322 by the poly(dG)·poly(dC) joining method. In the case of proinsulin, a fused protein bearing both insulin and β-lactamase antigenic determinants was produced and was carried outside of the cell by a 23-amino acid leader sequence in the β-lactamase (31). In the current studies, we independently used the same principle for the construction of clones that are potentially capable of synthesis and excretion of fused proteins that contain the ACTH or β-LPH peptide or both. It will thus be of interest to investigate the production of the ACTH-β-LPH precursor and its component peptides in the clones described here.

Note Added in Proof: Additional DNA sequence analysis confirms that plasmid pSNAC20 contains the entire coding sequence for β-LPH as well as for ACTH.

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