The administration of high levels of estrogen is a well-established method for producing prolactin-secreting pituitary tumors in rodents but the mechanism of tumor induction is not clear. In this paper we describe a cDNA clone (pEIC) which has been isolated from an estrogen-induced pituitary tumor cDNA library. The mRNA transcript corresponding to the pEIC clone is 0.9 kilobase in length and is not detectable in normal pituitaries but is expressed as early as 3 h after estrogen stimulation. Nucleotide sequence analysis of two 700-base pair recombinant clones shows that they encode a 124-amino acid protein which is 70% identical to the porcine galanin precursor. The sequence of 29 amino acid residues coded for by the pEIC cDNA clone is 88% identical with porcine galanin with only three amino acid substitutions near the C terminus. This extensive homology suggests that the pEIC cDNA clone codes for rat galanin or a protein belonging to the galanin gene family.

These results provide the first evidence of a physiological regulator (estrogen) of the expression of the galanin gene. They also imply that galanin is secreted by prolactin-secreting tumors. Because intracerebroventricular injection of galanin can stimulate prolactin secretion and galanin inhibits hypothalamic dopamine release, it is conceivable that galanin may play a role in the induction of prolactin-secreting tumors.

Prolactin-secreting tumors are the most common type of human pituitary tumor, accounting for almost half of all tumors of this gland (Kovacs and Horvath, 1985). The lactotroph, the PRL-secreting cell of pituitary, is highly responsive to the influence of E2 (Lieberman et al., 1982). During pregnancy there is massive hyperplasia of prolactin-secreting cells such that there is a 50% increase in the weight of the pituitary gland. It is recognized that a proportion of PRL-secreting tumors undergo rapid expansion during pregnancy, presumably due to the influence of increasing levels of E2 (Holmgren et al., 1982; Melmed et al., 1986) that are secreted during pregnancy. At present there is no way to identify those PRL-secreting tumors that will grow rapidly under the influence of E2 from those that will not, nor is it clear whether the effects of E2 on pituitary growth are direct or indirect. Evidence supporting the view that the proliferative effect of E2 is mediated by estromedins has been suggested by Sirbasku (1978) and Biro (1986). In order to explore the mechanism by which estrogen induces pituitary tumors we have examined the cDNA clones that are induced by estrogen in Fischer rats, a rat strain in which estrogen leads to the rapid development of pituitary tumor in a high proportion of the animals. In this paper we report the isolation and characterization of a cDNA clone that is induced by estrogens in normal pituitary glands and which is expressed at a high level in pituitary tumors induced by estrogens.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radioisotopes were purchased from Du Pont-New England Nuclear. Reverse transcriptase was from Life Sciences Inc. (St. Petersburg, FL). RNase A, T4 DNA polymerase, and some restriction enzymes were from Bethesda Research Laboratories. T4 DNA ligase, polynucleotide kinase, and some restriction enzymes were from Boehringer Mannheim. EcoRI methylase and Escherichia coli DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA). E. coli DNA polymerase (Klenow fragment) and packaging kits were from Amersham Corp. The 17-mer sequencing primer and oligo(dT)-cellulose (type 3) were supplied by Collaborative Research, Inc. (Wall- tham, MA). Protein A Sepharose, deoxynucleotides, deoxyribonucleotides, and EcoRI linkers were from Pharmacia LKB Biotechnology Inc. Nitrocellulose was from Schleicher and Schuell.

**Induction of Pituitary Tumors**—Female Fisher 344 rats (200-g weight) were obtained from Charles River (Quebec, Canada). Pituitary tumors were induced in rats using the alicyclic tubes containing the synthetic estrogen preparation, diethylstilbestrol, as reported previously (Vrontakis et al., 1987). For studies on the induction of an estrogen-inducible mRNA (pEIC) female rats were given 10 μg of 17-estradiol subcutaneously.

**Poly(A) mRNA Isolation**—Pituitaries were removed from Fisher 344 rats and frozen in liquid nitrogen. Total RNA was isolated by the guanidine thiocyanate/cesium chloride method (Chirgwin et al., 1979). Poly(A)+ mRNA was isolated by two cycles of oligo(dT)-cellulose chromatography (Aviv and Leder, 1972).

**Construction of cDNA Library**—A cDNA library was prepared from estrogen-induced pituitary tumor mRNA. The library was constructed in phage ϕgt 10, as described by Huynh et al. (1986) except that the second strand was made according to the protocol of Okayama and Berg (1982). DNA was packaged using an in vitro packaging kit from Amersham Corp. according to the supplier’s instructions. Recombinant plaque were plated on high frequency lysogen strain E. coli MA150 (Young and Davis, 1983). Phage plates were blotted onto nitrocellulose by the method of Benton and Davis (1977).

All recombinant DNA research was performed under the current Medical Research Council (Canada) guidelines for biohazardous materials.

**Screening of Pituitary cDNA Library**—To screen initially for cDNAs which were more strongly expressed in the pituitaries of E2-treated rats, an enriched cDNA probe from the E2-induced pituitary tumor mRNA was obtained by subtractive hybridization according to Davis et al. (1984). Plaques which hybridized strongly to this probe were picked and rescreened at a lower density. Differential screening of the library was followed with a [32P]cDNA probe made from control pituitary mRNA (−) and E2-induced pituitary tumor mRNA (+).

**RNA Blot Analysis**—Total RNA samples extracted for Northern blot analysis (Alwine et al., 1977) from pituitaries of control or estrogen-treated rats were size-fractionated on 1.3% agarose, 2.2 M formaldehyde gel (Lehrach et al., 1977) and blotted onto nitrocellulose.
paper (Thomas, 1980). Hybridizations were performed at 42 °C in 50% formamide using 10^6 dpm/ml of 32P-labeled pEIC cDNA insert nick-translated to a specific activity of 3−5 × 10^8 dpm/µg. Final washing conditions were 0.2 × SSC, 0.1% sodium dodecyl sulfate at 65 °C for 30 min.

DNA Sequence Determination—The pEIC clone was digested by restriction enzymes using the three-core buffer as described by Maniatis et al. (1982). Restriction enzyme fragments were sized either on agarose or polyacrylamide gels using HaeIII-cut φX174 fragments as markers. Appropriate restriction fragments were subcloned with the m13 vectors mp18 or mp19 (Vieira and Messing, 1982). Recombinant phage DNA was propagated in E. coli JM 101. DNA sequencing was by the dideoxy chain termination method (Sanger et al., 1977) using 35S-labeled dATP and the gradient gel modification (Biggin et al., 1982). The library was constructed in the phage vector XgtlO and expressed from E. coli JM 101. DNA sequencing was by the dideoxy chain termination method (Sanger et al., 1977) using 35S-labeled dATP and the gradient gel modification (Biggin et al., 1982). The gels were fixed in 10% acetic acid and dried before exposure to Kodak XAR film.

RESULTS

Isolation of the pEIC Clone—A cDNA library was constructed using mRNA from estrogen-induced pituitary tumors. The library was constructed in the phage vector λgt10 as described under "Experimental Procedures." The library was initially screened with an enriched cDNA probe also made from estrogen-induced pituitary tumor mRNA as described under "Experimental Procedures." Thirty clones that strongly hybridized to this probe were selected for further screening by differential hybridization with [32P]cDNA probes made from control pituitary mRNA and estrogen-induced pituitary tumor mRNA. The differential screening resulted in the isolation of three estrogen-induced and two estrogen-suppressed clones. From the three estrogen-induced clones, the pEIC was not detectable at all with the cDNA probe made from control pituitary mRNA. When the pEIC clone was used to rescreen a further 2200 recombinant plaques of the λgt10 library 11 more clones were isolated suggesting that the pEIC clone represents approximately 0.5% of the estrogen-induced pituitary tumor mRNA.

Expression of the pEIC in the Rat Pituitary—Fig. 1 shows autoradiograms of a Northern blot of RNA from normal female pituitaries and estrogen-induced pituitary tumors. Only the estrogen-induced pituitary tumor RNA hybridizes to the pEIC insert indicating that the RNA is not detectable in normal pituitaries. The transcript of the pEIC cDNA clone has a size of approximately 900 base pairs. As an internal control for the quality of the blotted RNA, hybridization of the same blot with the rat PRL cDNA probe indicates hybridization to RNA in both experimental conditions.

Time course of the induction of pEIC RNA by estrogen shown in Fig. 2 indicates that the transcript is detectable as early as 3 h after a single injection of the 17β-estradiol with the peak around 9 h and after 24 h has declined. The induction of the pEIC transcript is dose-dependent (data not shown).

DNA Sequence Analysis—Restriction enzyme analysis and sequencing strategy for the pEIC cDNA clones are shown in Fig. 3. Two clones, pEICA and pEICB, were analyzed to obtain the nucleotide sequence. The nucleotide sequence and the deduced amino acid sequence of pEIC clones are given in Fig. 4. The pEIC clone encodes a 124-residue protein which has a molecular weight of 13,313. The 3'-untranslated region is 184 base pairs in length and contains an AATAAA polyadenylation signal (Proudfoot and Brownlee, 1976) 12 nucleotides upstream from the poly(A) tail. Comparison of the pEIC cDNA clone sequence with the sequences in Genbank revealed that 70% of this sequence at the amino acid level is identical to the porcine galanin precursor (Rokaeus and Brownstein, 1986). Fig. 5 shows a comparison of the porcine amino acid galanin sequence with the predicted amino acid sequence of the pEIC cDNA clone. The 29-amino acid single porcine galanin sequence is 88% identical with the pEIC sequence with a serine instead of histidine at residue 55, histidine instead of tyrosine at residue 58, and threonine instead of alanine at residue 61. The pEIC sequence has an extra valine at residue 87, so the total amino acid sequence consists of 124 residues instead of 123 as is the case for the porcine galanin
Fig. 4. Nucleotide and amino acid sequence of pEIC cDNA. Nucleotides are numbered at the end of lines and amino acids are numbered throughout. An open reading frame begins at the first ATG (nucleotides 125-127) and continues to the stop codon TGA (nucleotides 497-499). The closed box corresponds to the galanin sequence. The polyadenylation site is indicated ( ).

Fig. 5. Comparison of the amino acid sequence of the pEIC cDNA clone and galanin precursor. The lower line corresponds to the amino acid sequence of the pEIC cDNA clone. The upper line is the porcine galanin precursor (Rokaeus and Brownstein, 1986). The different amino acid residues in the pEIC cDNA clone are underlined. The closed box corresponds to the galanin sequence.

DISCUSSION

We have isolated and sequenced a cDNA clone from estrogen-induced pituitary tumor mRNA. The sequence of the pEIC clone indicates that 70% of the amino acid sequence is identical to porcine galanin precursor, with all but 3 of 29 amino acid residues (88%) corresponding to the porcine galanin sequence, indicating that the pEIC cDNA clone is either the rat equivalent of galanin or less probably a sequence belonging to the galanin gene family.

The mRNA coding for the pEIC cDNA clone is approximately 900 base pairs, the same size that has been reported for galanin (Rokaeus and Brownstein, 1986). The transcript of the pEIC cDNA clone is not detectable at all in normal pituitaries and is strongly induced by estrogen in the pituitary. The induction of the transcript occurs as early as 3 h after estrogen administration and is a primary event since administration of cycloheximide 10 min before the estrogen administration does not inhibit the induction of the transcript. After 7 weeks of treatment with estrogen the pEIC cDNA clone transcript is quite abundant in the pituitary representing 0.5% of the pituitary mRNA.

Galanin was first identified as a gastrointestinal neuropeptide (Tatemoto et al., 1983), and subsequent studies demonstrated that it is localized to nerves of the myenteric and submucous plexus (Melander et al., 1985). Galanin-like immunoreactivity has also been found in the central nervous system (Ching et al., 1985) with the highest concentration in the median eminence and hypothalamus. The role of galanin in the central nervous system is unknown. Its presence in the median eminence suggests that it may play a role in the regulation of anterior pituitary function. It has been reported that intracerebroventricular injection of galanin into the rat brain stimulates PRL and growth hormone release (Ottlecz et al., 1986; Koshiyama et al., 1987) possibly by stimulating vasoactive intestinal polypeptide secretion. Nordstrom et al. (1987) reported that galanin has an inhibitory effect on dopamine release from the rat median eminence, a finding which would support a role for galanin in the regulation of PRL secretion as dopamine itself is a potent inhibitor of PRL secretion.

Since administration of estrogen in rodents induces prolactin-secreting pituitary tumors and as we have shown in this paper estrogen administration induces a high level of expression of galanin mRNA in the pituitary, it is conceivable that estrogen regulates PRL secretion and or pituitary tumor formation through a step that involves galanin.

Further studies are in progress to elucidate the exact mechanism by which estrogen regulates galanin and PRL synthesis and the role of galanin for the formation of PRL-secreting pituitary tumor by estrogen.

REFERENCES

Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5350-5354
Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408-1412
Benton, W. D., and Davis, R. W. (1977) Science 196, 180-182
Biggin, M. D., Gibson, T. J., and Hong, G. F. (1977) Proc. Natl. Acad. Sci. U. S. A. 80, 3963-3966
Biro, J. C. (1986) Medical Hypotheses 19, 199-228
Ching, J. L., Christofides, N. D., Aman, P., Gibson, S. J., Allen, Y. S., Su, H. C., Tamemoto, K., Morisson, J. F. B., Polak, J. M., and Bloom, S. J. (1985) Neuroscience 16, 343-354
Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
Davis, M. M., Cohen, D. I., Nielsen, E. A., Steinmetz, M., Paul, W. E., and Hood, L. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2194-2198
Holmgren, U., Bergstrand, G., Hagenfeldt, K., and Warner, S. (1982) Acta Endocrinol. 111, 455-459
Huynh, T. V., Young, R. A., and Davis, R. W. (1985) in DNA Cloning: A Practical Approach (Glover, D. M., ed) Vol. I, pp. 49-77, IRL Press, Oxford

2 M. E. Vrontakis, unpublished data.
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Koshiyama, H., Kato, Y., Inoue, T., Murakami, Y., Ishikawa, Y., Yanaihara, N., and Imura, H. (1987) Neurosci. Lett. 75, 49-54

Kovacs, K., and Horvath, E. (1985) in Morphology of Adenohypophysial Cells and Pituitary Adenomas (Imura, H., ed) pp. 25-55, Raven Press, New York

Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) Biochemistry 16, 4743-4751

Lieberman, M. E., Maurer, R. A., Claude, P., and Gorski, J. (1982) Mol. Cell. Endocr. 25, 277-294

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Melander, T., Hokfelt, T., Rokaeus, A., Fahzenkrug, J., Tatemoto, K., and Mutt, V. (1985) Cell Tissue Res. 239, 253-270

Melmed, S., Braunstein, G. D., Chang, R. J., and Becker, D. P. (1986) Ann. Intern. Med. 105, 238-253

Nordstrom, O., Melander, T., Hokfelt, T., Bazfai, T., and Goldstein, M. (1987) Neurosci. Lett. 73, 21-26

Okayama, H., and Berg, A. (1982) Mol. Cell. Biol. 2, 161-170

Ottlecs, A., Samson, W., and McCann, S. M. (1986) Peptides 7, 51-53

Proudfoot, N. J., and Brownlee, G. G. (1976) Nature 263, 211-214

Rokaeus, A., and Brownstein, M. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6287-6291

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467

Sribasaku, D. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3786-3791

Tatemoto, K., Rokaeus, A., Jornvall, H., McDonald, T. J., and Mutt, V. (1983) FEBS Lett. 164, 124-128

Thomas, P. C. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201-5205

Vieira, J., and Messing, J. (1982) Gene (Amst.) 19, 259-268

Vrontakis, M. E., Thiliveris, J. A., and Friesen, H. G. (1987) J. Endocrinol. 113, 383-388

Young, R. A., and Davis, R. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1194-1198