Pro-opiomelanocortin mRNA size heterogeneity in ACTH-dependent Cushing's syndrome

A. J. L. Clark, P. M. Lavender, G. M. Besser and **L. H. Rees**

Departments of Chemical Endocrinology and Endocrinology, Medical College of St Bartholomew's Hospital, London EC1A 7BE

RECEIVED 20 June 1988

ABSTRACT

As an approach to understanding the abnormalities of pro-opiomelanocortin (POMC) gene regulation in human ACTH-secreting tumours, we have analysed the POMC mRNA content of nine such tumours using the Northern blot technique. Most of the tumours and normal human pituitary contained easily detectable quantities of POMC mRNA. The length of this message in most tumours was similar to, or slightly larger than, that in the normal pituitary (1150–1200 bases). Ribonuclease H studies suggested that the origin of any size heterogeneity was a longer poly(A) tail in the tumour RNA. Some tumours, however, expressed a short POMC mRNA (800 bases) which may lack the first two exons of the POMC gene as has been described. A third POMC mRNA size variant (1500 bases) was also seen in low levels in two cases, and as the principal mRNA species in one case. Primer extension and S1 nuclease protection studies suggested that most transcripts in the tumours analysed originated from the conventional promoter, and thus the use of an alternative promoter is not an adequate explanation for the expression of this gene in ectopic ACTH-secreting tumours.

Journal of Molecular Endocrinology (1989) **2**, 3–9

INTRODUCTION

Adrenocorticotrophin (ACTH)-dependent Cushing's syndrome is characterized by inappropriate expression of the pro-opiomelanocortin (POMC) gene either within an anterior pituitary corticotroph adenoma, or in a non-pituitary tumour—the so-called ectopic ACTH syndrome. The normal inhibition of expression of this gene by glucocorticoids is partially or completely lost in these circumstances. This suggests that there may be either a defect in the mechanism of glucocorticoid suppression, or that a more potent positively acting transcriptional effect exists. An hypothesis that could explain these observations is that the POMC gene in these tumours is driven by an alternative promoter which lacks the tissue-specific and regulatory constraints of the POMC promoter. As an approach to examining this hypothesis we have used the techniques of DNA/RNA hybridization to examine the fidelity of POMC gene transcription in such tumours.

This approach has been used by others in tumours from patients with the ectopic ACTH syndrome. Tsukada, Nakai, Jingami *et al.* (1981) reported the existence of a normal length and a longer POMC mRNA in a thymic carcinoid. *In-vitro* translation of these RNAs produced a single 38 kDa peptide, suggesting that the longer mRNA did not contain any additional amino acid coding sequence.

DeBold, Schwarer, Connor *et al.* (1983) found a normal (1100 base) and a large (1300 base) mRNA in a pancreatic metastasis from a thymic carcinoid, and reported some DNA sequence of a POMC cDNA derived from this source. This sequence showed that the 3′ end corresponded to that predicted from the human gene sequence, and that the transcriptional stop site was correctly placed.

Steenbergh, Hoppener, Zandberg *et al.* (1984) demonstrated a POMC mRNA of 1300 bases in metastases from two medullary carcinomas of the thyroid, and found by DNA sequencing of a cDNA clone derived from one of these that the 3′ end of this mRNA corresponded to the normal. The same group have more recently reported the finding of a large mRNA in phaeochromocytoma and adenocarcinoma of the lung (Hoppener, Steenbergh, Mooren *et al.* 1986). Finally, de Keyzer, Bertagna, Lenne *et al.* (1985) have described a thymic carcinoid tumour that produces a normal and a large (~1450 bases) mRNA. We have now studied a larger group of

  Printed in Great Britain
0952-5041/89/002 003 $02.00/0

ACTH-producing tumours, including one from the pituitary.

MATERIALS AND METHODS

Tumour collection

Tumours were collected at surgery or post-mortem (for tumour 2 there was a 60-h delay between death and autopsy) from nine patients with Cushing's syndrome, eight with proven ectopic ACTH syndrome and one with Cushing's disease resulting from a basophil adenoma of the pituitary. Details of these tumours are shown in Table 1. Samples were divided into material for histopathological analysis and material for research studies, the latter being snap-frozen in liquid nitrogen or solid CO_2/ethanol. This tissue was subsequently stored at −70°C. Normal pituitaries were obtained from the National Pituitary Collection of normal post-mortem pituitary tissue which had been stored at −70°C.

RNA preparation

Total cellular RNA was prepared by the method of Chirgwin, Przbyla, MacDonald & Rutter (1979), in which tissue was homogenized in guanidine isothiocyanate and then centrifuged through a caesium chloride cushion. The RNA precipitate was then resuspended in 10 mM Tris–HCl (pH 7·5) containing 1 mM EDTA and extracted with chloroform/isobutanol (4:1, v/v) before ethanol precipitation. RNA was quantified by measurement of the optical density at 260 nm.

Northern blots

Total cellular RNA was separated by electrophoresis (2 V/cm for 15 h) on 1·4% (w/v) agarose/formaldehyde (6·5%, v/v) gels run in 20 mM 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (pH 7·0). RNA samples were denatured before loading by heating to 65°C in 33% formamide/5% formaldehyde/20 mM MOPS, cooling on ice and adding glycerol and dyes. After electrophoresis, gels were stained with ethidium bromide (5 μg/ml), destained to visualize the 18S and 28S RNA bands and then transferred to nitrocellulose paper in 20 × SSC (1 × SSC is 0·15 M sodium chloride and 0·015 M sodium citrate; pH 7·0). The nitrocellulose was baked in a vacuum oven (80°C for 2 h) and prehybridized and hybridized as described previously (Thomas, 1980) in deionized formamide (50%), 5 × SSC, sodium phosphate buffer (50 mM; pH 6·8), sheared salmon sperm DNA (300 μg/ml), sodium dodecyl sulphate (0·05%) and 5 × Denhardt's solution (50 × Denhardt's is ficoll (1%, w/v), polyvinylpyrrolidone (1%, w/v) and bovine serum albumin (1%, w/v)).

The probe used was the full length bovine POMC cDNA insert from the plasmid pSNAC20 (Nakanishi, Inoue, Kita *et al.* 1979). This has extensive sequence homology with the human POMC gene. This was labelled with [^{32}P] dCTP (Amersham International, Amersham, Bucks, U.K.) to high specific activity (10^9 c.p.m./μg) by the oligonucleotide-primed labelling technique (Feinberg & Vogelstein, 1983), and approximately 10^7 c.p.m. were used per filter. After 24-h hybridization at 42°C, blots were washed in 0·2 × SSC at 55°C and exposed to Kodak XAR-5 film with intensifying

TABLE 1. Summary of the human ACTH-producing tumours studied, and the size(s) of the POMC mRNAs they contained

No.	Histology	RNA size (bases)	Comment
1	Medullary carcinoma of the thyroid	1500	No metyrapone pre-surgery. Low ACTH
2	Poorly differentiated pancreatic tumour	900 + 700	Post-mortem specimen. ACTH immunostaining negative
3	Pulmonary carcinoid	1200	Atypical histological features
4	Pulmonary carcinoid	1150–1300	
5	Pulmonary carcinoid	1250	'Big' ACTH on chromatography*
6	Pulmonary carcinoid	1250	
7	Pancreatic carcinoid (liver metastasis)	800	
8	Basophil adenoma of the pituitary	1150–1200	
9	Normal pituitary	1150–1200	
10	Normal pituitary	1150–1200	
11	Tumour 6—alternative specimen	1200 + 1500	
12	Pulmonary carcinoid	1250 + 1500	'Big' ACTH on chromatography

* See Hale, Besser & Rees (1986).

screens at −70°C for various intervals, usually 24–48 h.

RNA size markers

A frequent problem with Northern blot experiments lies in estimating the size of RNA bands detected. We therefore synthesized a series of RNA transcripts using the bovine POMC cDNA cloned into the polylinker Pst I site of pT712 (Gibco BRL, Paisley, Strathclyde, U.K.), a T7 polymerase vector. By cutting the plasmid with certain restriction enzymes, i.e. Pvu II, Bam HI, Eco RI (New England Biolabs, Beverly, MA, U.S.A.), and then transcribing with T7 polymerase (Gibco BRL) we were able to generate RNA transcripts of 1353, 1150 and 665 bases in length respectively. These were electrophoresed in parallel with test RNA samples and then transferred to nitrocellulose, following which they were detectable by hydridization to the POMC cDNA probe.

Sizes of test RNA species were determined by plotting the regression line relating the RNA size marker ($\log_{10}$ size in bases) to the distance migrated through the gel (in cm) and reading the migration distance of the hybridizing band of interest against this.

RNAse H technique

RNA samples from three tumours (Nos 1, 3 and 12 in Table 1) and normal pituitary were annealed to synthetic oligo(dT) (400:1 molar excess of oligo(dT)) in 100 mM KCl by heating to 65°C for 2 min, and then incubating at 37°C for 30 min. To this were then added 10 × RNAse H buffer (1 × = 20 mM Tris–HCl (pH 7·5), 10 mM magnesium chloride, 0·1 mM dithiothreitol and 5% (w/v) sucrose) and 2 units RNAse H (Gibco BRL). Incubation was for 30 min at 37°C. This was then extracted with phenol/chloroform and precipitated with ethanol. The products were analysed by Northern blot analysis, each sample being run alongside an untreated sample of the same RNA. Nitrocellulose filters were hybridized to the bovine POMC cDNA probe as before, revealing the sizes of the poly(A)-tailed and -deficient RNA species.

S1 nuclease protection

The 173 bp Ava I/Ava I fragment of the 5′ end of the human POMC gene was prepared by acrylamide gel electrophoresis and electroelution, and was then ^{32}P 5′ end-labelled using [^{32}P]ATP (Amersham International) and T4 polynucleotide kinase (Gibco BRL). This probe was then incubated with RNA as described previously (Maniatis, Fritsch & Sambrook, 1982), initially at 75°C for 5 min and then at 60°C for 3 h. This relatively high temperature was chosen in view of the 67% GC content of this DNA fragment. Subsequently, 300 µl S1 nuclease buffer

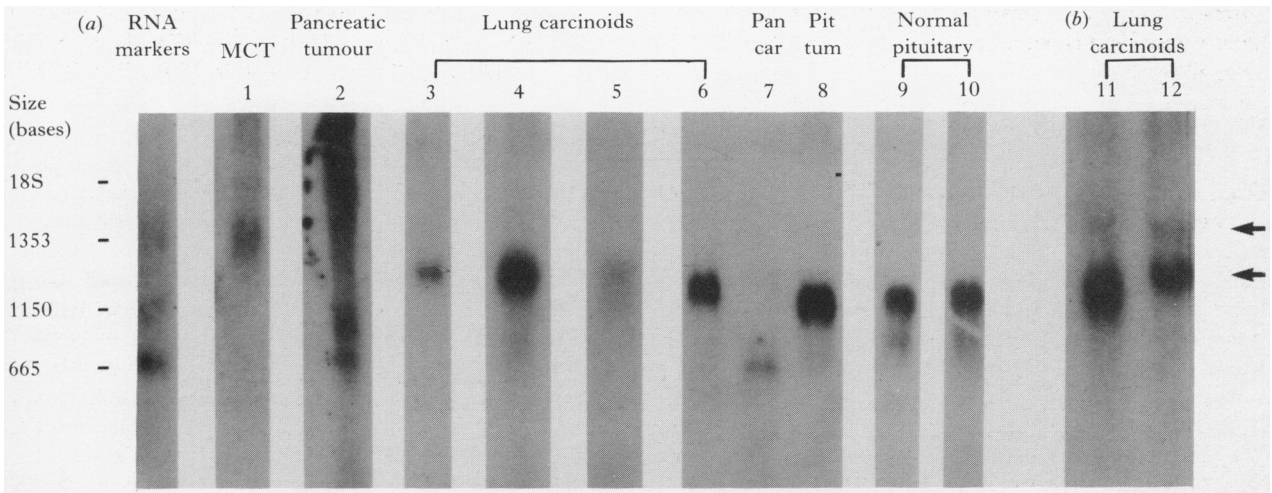

FIGURE 1. (*a*) Autoradiographs of Northern blots of total cellular RNA (10 µg per lane) from several human ectopic ACTH-producing tumours separated by electrophoresis, transferred to introcellulose and hybridized with the ^{32}P-labelled bovine POMC cDNA probe. Size markers are based on the 18S ribosomal RNA and three synthetic POMC RNA markers (see text for details). (*b*) Autoradiographs of Northern blots of two RNAs which emphasise the larger molecular weight species (upper arrow). MCT, medullary carcinoma of the thyroid; Pan car, pancreatic carcinoid; Pit tum, pituitary tumour.

were added with or without 300 units S1 nuclease (Gibco BRL). This was incubated for 30 min at 37°C. The reaction was then stopped and extraction performed once with phenol/chloroform, before precipitation with ethanol. The sizes of the protected fragments were then analysed by electrophoresis on denaturing 8% (w/v) polyacrylamide gels and autoradiography.

Primer extension

A synthetic 20 base DNA primer complementary to nucleotides +26 to +46 of the human POMC sequence was prepared, and ^{32}P labelled at its 5′ end with T4 polynucleotide kinase and [^{32}P]ATP (Amersham International). Unincorporated [^{32}P]ATP was separated by one passage over a 1 ml Sephadex G-50 column (Pharmacia, Uppsala, Sweden). This primer was annealed to RNA in 100 mM sodium chloride, 20 mM Tris–HCl (pH 8·0), 1 mM EDTA at 60°C overnight. Samples were then precipitated with ethanol and resuspended in reverse transcriptase buffer (50 mM Tris–HCl (pH = 8·3), 50 mM potassium chloride, 6 mM magnesium chloride and 10 mM dithiothreitol) containing 2·5 mM unlabelled deoxynucleotide triphosphates and 20 μg actinomycin D/ml. MMLTV reverse transcriptase (400 units; Gibco BRL) was added to this, and incubation was for 60 min at 37°C. RNA was then denatured by a 30-min incubation in 0·2 M sodium hydroxide, followed by phenol/chloroform extraction and ethanol precipitation. Single-stranded extended DNA fragments were then analysed by electrophoresis on denaturing 8% polyacrylamide gels and autoradiography.

RESULTS

Total cellular RNA from the nine tumours and two normal pituitaries was analysed by the Northern blot procedure. (Fig. 1). In all tumours a band could be identified. The intensity of this band probably reflects both the variable level of POMC gene expression and the partially degraded nature of some of the RNA samples. Some size heterogeneity is present in this 'normal' mRNA (cf. lanes 6 and 8, Fig. 1). In cases two and seven a shorter RNA species of 700–900 bases was found. A larger band of about 1500 bases can be seen in lanes 1, 11 and 12, in the latter two cases co-existing with a 'normal' sized band. The sizes of all bands as calculated from the RNA size markers are summarized in Table 1.

A variety of reasons might underly this heterogeneity of POMC mRNA. The larger variants could be the result of alternative splicing of one of the two introns, an upstream transcriptional start site, or a longer 3′ end. Alternative splicing of the second intron of the gene seems unlikely, since this would either interfere with or add to the amino acid sequence of the POMC peptide. The use of a second poly(A) addition signal is also an unlikely explanation, since although there are two AATAAA sequences at the 3′ end of the gene, they are within 30 bp of each other, and thus use of the alternative signal would contribute little to the overall length of the mRNA.

One possibility is that the poly-(A) tail may differ in length between pituitary and tumour POMC mRNAs. This was investigated by analysing several tumour and normal pituitary RNAs before and after annealing to synthetic oligo(dT) and treatment with RNAse H. This procedure selectively removes the poly(A) tract, thus permitting a measurement of its length. Results suggest that the pituitary POMC mRNA has a poly(A) tail of ~150 bases (Fig. 2). Tumour 3 contained RNA which had the same poly(A) tail length as normal pituitary, whereas tumour 12 appeared to have a longer poly(A) tail. The large POMC mRNA from tumour 1 shortened after RNAse H treatment, but the final product was still longer than the treated message in other lanes, implying an additional cause for its larger size.

The 5′ end of the mRNA was next studied, initially using the S1 nuclease protection technique. A 173 bp Ava I/Ava I DNA fragment derived from the human POMC gene was used as the S1 probe after labelling at its 5′ end with ^{32}P. This probe extends 93 bp upstream of the conventional POMC transcriptional start site. With several of the RNAs tested the quality and quantity of material available was insufficient to obtain a satisfactory analysis. Several samples were, however, successfully examined, and all showed the result exemplified in Fig. 3, suggesting that most of the RNA starts at a site corresponding to +1. A very small proportion of the RNA, perhaps <5%, probably starts from a site further upstream.

This finding can be independently assessed using the technique of primer extension. Using a synthetic DNA primer complementary to the sequence between positions +26 and +46, extended products shown in Fig. 4 were obtained. This demonstrates that the major transcriptional start sites are at +1 and +2 (both A residues). Three minor start sites are located at positions approximating to −83, −88 and −94. There was also a barely visible extended primer at 780 bases, suggesting a transcriptional start site at −734. This latter feature was unique to the ectopic ACTH tumour RNAs, whereas the other alternative start sites were found to a degree in 'normal' pituitary RNA.

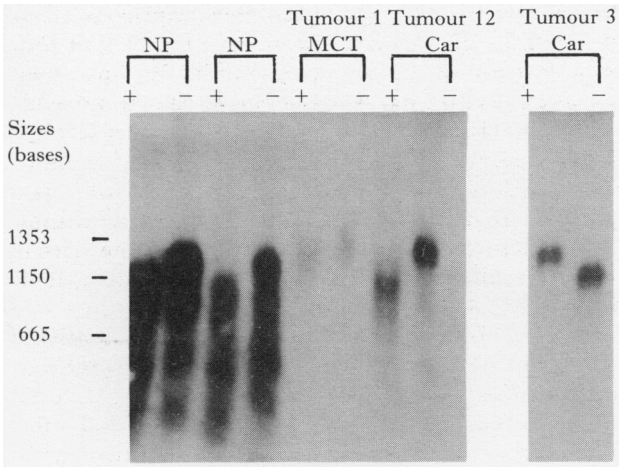

FIGURE 2. Autoradiographs of Northern blots of RNA samples from two normal pituitaries and from tumours 1, 3 and 12 treated with (+) or without (−) RNAse H. There is significant degradation of the pituitary RNAs since these were obtained from post-mortem specimens. Size markers are based on synthetic RNA markers. NP, normal pituitary; MCT, medullary carcinoma of the thyroid; Car, lung carcinoid.

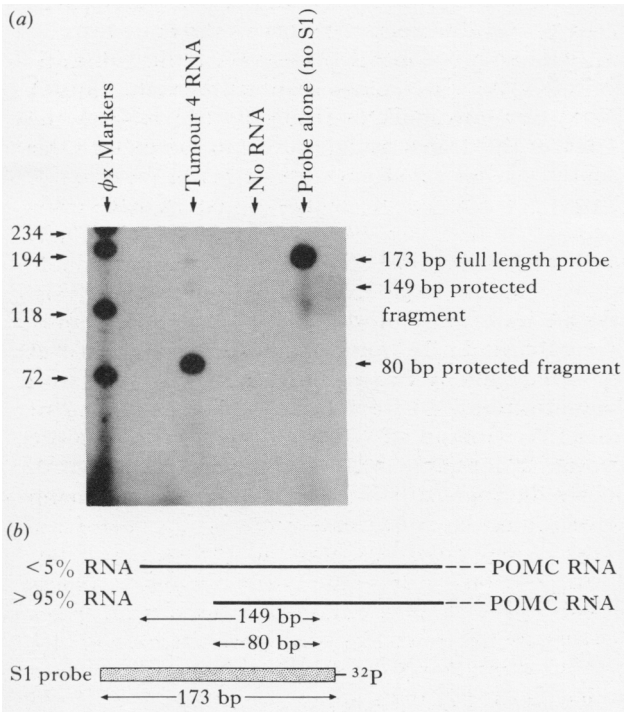

FIGURE 3. (*a*) S1 nuclease protection experiment using RNA from tumour 4 in Fig. 1. The size of the undigested probe and the protected fragments are indicated. (*b*) Diagrammatic representation of the findings suggested by this experiment. In this RNA sample over 95% of transcripts appear to initiate at +1.

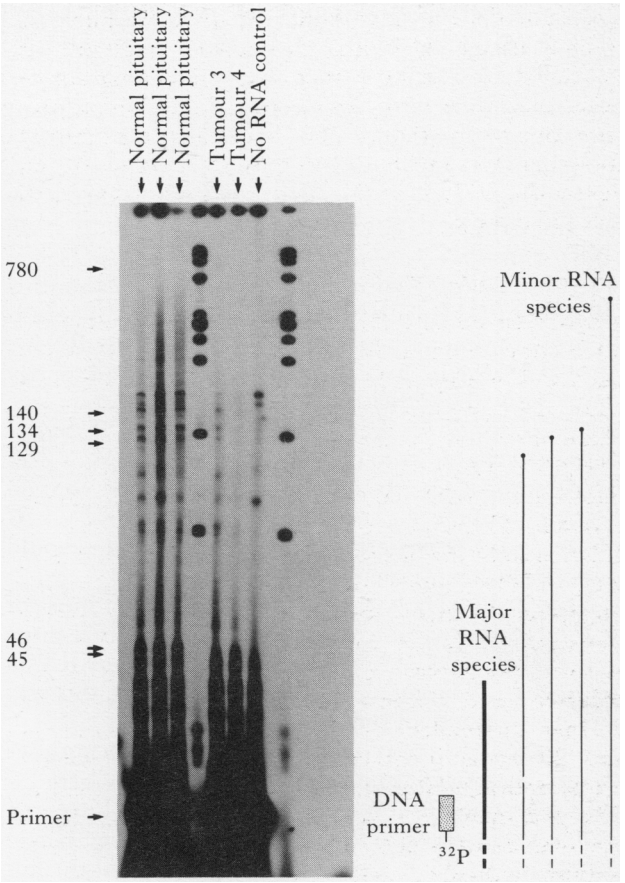

FIGURE 4. (*a*) Primer extension experiment using RNAs from two human tumour samples and three 'normal' pituitaries. The primer and the normally initiated transcripts (at +1 and +2) cannot be clearly identified on this exposure, but are clearly seen on shorter exposures. The extended products and their estimated sizes are indicated by the arrows on the left. Note, there is significant background in the 'no RNA' lane in this experiment. (*b*) Diagrammatic representation of the various RNA species and their relationship to the oligonucleotide probe.

DISCUSSION

The origin of the ectopic humoral syndromes has been the subject of much discussion and debate (Rees, 1975; Baylin & Mendelsohn, 1980; Pearse, 1980; Stevens & Moore, 1983). There is evidence that cells that produce POMC, or at some stage in differentiation have the capability of producing POMC, are widespread in man (Krieger, 1983). Thus current views include those that a process of either de-differentiation or dys-differentiation of a cell derived from an endocrine cell lineage has taken

place, either before or after neoplastic transformation of that cell. Any such theory has to take into account a number of phenomena that characterize the ectopic ACTH syndrome, e.g. the failure of POMC production by such tumours to be inhibited by glucocorticoids.

Another phenomenon that has been reported by several groups (Tsukada *et al.* 1981; DeBold *et al.* 1983; Steenbergh *et al.* 1984; de Keyzer *et al.* 1985; Hoppener *et al.* 1986) is the existence of an abnormally long POMC mRNA in these tumours. There is some variation in the reported length of these RNAs, probably partly because they do vary and partly because of the difficulties in obtaining accurate size markers for RNA gels. The use of SP6/T7 polymerase technology to obtain RNA transcripts of precisely known length, and which, since they contain POMC sequences, can hybridize to the POMC probe after transferring to nitrocellulose should overcome this problem.

Our results show that the mRNA size in most of the tumours was similar to that of the normal pituitary (1150–1200 bases). Some of the tumour RNAs were slightly larger and, in at least one case, this was because of a longer poly(A) tail.

Long poly(A) tails have been reported in a number of circumstances. The exact role of the poly(A) tail is not clear, but one apparent function is to stabilize the mRNA to which it is attached (Marbaix, Huez, Burns *et al.* 1975). There is a suggestion that the tail is progressively degraded during the lifetime of the RNA molecule. An unusually long poly(A) tail has been reported in rat hypothalamic POMC mRNA in contrast to that in the normal pituitary (Jeannotte, Burbach & Drouin, 1988). Long poly(A) tails have also been described on insulin mRNA in developing rat islet cells (Philippe, Chick & Habener, 1987) and also in fetal rat pancreas (Guggenheim & Permutt, 1986). The implications of this phenomenon in human POMC-secreting tumours are not clear.

S1 nuclease and primer extension experiments were undertaken to investigate the possibility that an alternative transcriptional start site was functional. The results show fairly conclusively that in the samples analysed, 95% of transcription initiates at the conventional start sites. Unfortunately, RNA of sufficient quantity or quality was not available to obtain adequate data from the tumours in which Northern analysis suggested a long RNA species. These findings suggest that the attractive hypothesis of a promoter unique to ACTH-producing tumours is untenable.

Thirdly, we have identified a small mRNA in tumours. This probably corresponds to the small POMC mRNA that has been reported by Lacaze-Masmonteil, de Keyzer, Luton *et al.* (1987) in human testis. This message results from transcription which probably initiates from a CG box promoter at the 3′ end of intron 2. Such a transcript should lack a signal sequence, and thus one would predict that the resultant peptide (if this mRNA is translated) would not be secreted into the endoplasmic reticulum, would consequently not gain access to the secretory vesicle and so would presumably not be processed. Whether this peptide would be secreted from the cell is not clear. However, since all these patients were selected because they had clinically apparent Cushing's syndrome, it would seem that ACTH was being secreted from some part of the tumour.

In conclusion, we have demonstrated that most clinically relevant ACTH-secreting tumours contain a POMC mRNA that is approximately the same size as that in the normal pituitary. Some minor size heterogeneity may result from variation in the length of the poly(A) tail. A long POMC mRNA has been identified and is of similar size to that described by others. This may result from transcription initiating at an upstream site. A weak upstream promoter is probably active in all tumours and in the normal pituitary, and this may function more actively in circumstances of glucocorticoid excess. In the one patient whose Cushing's syndrome was not controlled with metyrapone before removal of the tumour, a long POMC mRNA was seen in isolation. Thirdly, some tumours make a small POMC mRNA, but whether this transcript gives rise to secreted POMC peptides is not yet clear.

ACKNOWLEDGEMENTS

We are most grateful to the physicians who referred the patients to us from whom these tumours were removed, and to those who permitted us to study tumours removed from patients whilst under their care. We should also like to thank Professor S. Numa, Kyoto, Japan, for permitting us to use the plasmid pSNAC20. This work was supported by the Medical Research Council.

REFERENCES

Baylin, S. B. & Mendelsohn, G. (1980). Ectopic (inappropriate) hormone production by tumors: mechanisms involved and the biological and clinical implications. *Endocrine Reviews* **1**, 45–77.

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

DeBold, C. R., Schwarer, M. E., Connor, T. B., Bird, R. E. & Orth, D. N. (1983). Ectopic pro-opiomelanocortin: sequence

of cDNA coding for β-malanocyte stimulating hormone and β-endorphin. *Science* **220**, 721–723.

Feinberg, A. P. & Vogelstein, B. (1983). A technique for labelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.

Guggenheim, N. Z. & Permutt, M. A. (1986). Identical transcription initiation sites for proinsulin messenger RNA in three insulin expressing tissues. *Endocrinology* **118**, 1710–1715.

Hale, A. C., Besser, G. M. & Rees, L. H. (1986). Characterization of pro-opiomelanocortin-derived peptides in pituitary and ectopic adrenocorticotrophin-secreting tumours. *Journal of Endocrinology* **108**, 49–56.

Hoppener, J. W. M., Steenbergh, P. H., Mooren, P. J. J., Wagenaar, S. S., Jansz, H. S. & Lips, C. J. M. (1986). Detection of mRNA encoding calcitonin, calcitonin gene related peptide and proopiomelanocortin in human tumors. *Molecular and Cellular Endocrinology* **47**, 125–130.

Jeannotte, L., Burbach, J. P. H. & Drouin, J. (1988). Unusual proopiomelanocortin ribonucleic acids in extrapituitary tissues: intronless transcripts in testes and long poly(A) tails in hypothalamus. *Molecular Endicrinology* **1**, 749–757.

de Keyzer, Y., Bertagna, X., Lenne, F., Girard, F., Luton, J.-P. & Kahn, A. (1985). Altered proopiomelanocortin gene expression in adrenocorticotropin producing non-pituitary tumors. *Journal of Clinical Investigation* **76**, 1892–1898.

Krieger, D. T. (1983). The multiple faces of pro-opiomelanocortin, a prototype precursor molecule. *Clinical Research* **31**, 342–353.

Lacaze-Masmonteil, T., de Keyzer, Y., Luton, J.-P., Kahn, A. & Bertagna, X. (1987). Characterization of proopiomelanocortin transcripts in human nonpituitary tissues. *Proceedings of the National Academy of Sciences of the U.S.A.* **84**, 7261–7265.

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

Marbaix, G., Huez, G., Burns, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudd, U. & Littauer, U. Z. (1975). Absence of polyadenylation segment in globin messenger RNA accelerates its degradation in *Xenopus* oocytes. *Proceedings of the National Academy of Sciences of the U.S.A.* **72**, 3065–3067.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N. & Numa, S. (1979). Nucleotide sequence of cloned cDNA for bovine corticotrophin—β-lipotrophin precursor. *Nature* **278**, 423–427.

Pearse, A. G. E. (1980). The APUD concept and hormone production. *Clinics in Endocrinology and Metabolism* **9**, 211–222.

Philippe, J., Chick, W. L. & Habener, J. F. (1987). Multipotential phenotypic expression of genes encoding peptide hormones in rat insulinoma cell lines. *Journal of Clinical Investigation* **79**, 351–358.

Rees, L. H. (1975). The biosynthesis of hormones by non-endocrine tumours—a review. *Journal of Endocrinology* **67**, 143–175.

Steenbergh, P. H., Hoppener, J. W. M., Zandberg, J., Roos, B. A., Jansz, H. S. & Lips, C. J. M. (1984). Expression of the proopiomelanocortin gene in human medullary thyroid carcinoma. *Journal of Clinical Endocrinology and Metabolism* **58**, 904–908.

Stevens, R. E. & Moore, G. E. (1983). Inadequacy of APUD concept in explaining production of peptide hormones by tumours. *Lancet* 1, 118–119.

Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 5201–5205.

Tsukada, T., Nakai, Y., Jingami, H., Imura, H., Taii, S., Nakanishi, S. & Numa, S. (1981). Identification of the mRNA coding for the ACTH–β-lipotrophin precursor in a human ectopic ACTH producing tumor. *Biochemical and Biophysical Research Communications* **98**, 535–540.