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Ectopic synthesis of high-M_r calcitonin by the BEN lung carcinoma cell line reflects aberrant proteolytic processing

John H. Riley, Mark R. Edbrooke* and Roger K. Craig*+

Cancer Research Campaign Endocrine Tumour Molecular Biology Group and Medical Molecular Biology Unit, Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, Mortimer Street, London W1P 7PN, England

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Cloning and nucleotide sequence analysis of the human calcitonin mRNA from the BEN lung carcinoma cell line, a cell line known to secrete high-M_r forms of calcitonin, showed no difference in the coding region at the nucleotide level compared with calcitonin mRNA isolated from medullary thyroid carcinoma which secretes calcitonin monomer. Therefore, the secretion of high-M_r forms of calcitonin reflects the absence or limited activity of proteolytic processing enzymes within the secretory pathway of this cell line. In all other respects, as judged by RNA blotting and S_1 mapping, calcitonin/α-CGRP expression was identical to that found in medullary thyroid carcinoma, including the differential use of an alternative splice donor site within intron 1. The BEN cell line also produces low levels of α-CGRP mRNA and secretes CGRP antigenic determinants. Analysis of plasma CGRP levels in 12 patients with anaplastic lung carcinoma showed elevated levels in 11 of these, suggesting that CGRP may be an important diagnostic marker for this disease.

Calcitonin *Lung carcinoma* *Medullary thyroid carcinoma* *Proteolytic processing* *RNA splicing* *Calcitonin gene-related peptide* *Ectopic synthesis*

1. INTRODUCTION

Studies on the expression of the rat and human calcitonin gene have demonstrated the generation, via post-transcriptional processing events, of two distinct mRNA species encoding polyproteins which contain either calcitonin or the α-calcitonin gene-related peptide (α-CGRP) [1,2]. A second gene encoding human β-CGRP has also been described [2,3]. Calcitonin is normally synthesized by the C-cells of the thyroid (see [4]) and CGRP is co-produced by the same cells [5]. Discrete CGRP-producing cells are also found in the thyroid [5] and widely distributed in the central and peripheral nervous system [5]. Calcitonin and CGRP are synthesized as precursor polyproteins, which are cleaved and then amidated prior to secretion [7,8]. Calcitonin biosynthesis also occurs ectopically in non-thyroidal tumours including lung [9,10] and phaeochromocytomas [11]. Many such tumours secrete immunoreactive calcitonin of high M_r and little of the monomer (M_r 3500) [12,13]. We have previously described the ectopic expression of the calcitonin/α-CGRP gene in two small (oat) cell lung carcinoma cell lines, as judged by S_1 nuclease mapping and cDNA hybridisation analysis [2]. Here we examine by cDNA cloning, RNA hybridisation analysis, S_1 mapping and radioimmunoassay, the expression of the human calcitonin α-CGRP gene in a human large-cell bronchial carcinoma cell line (BEN), a cell line known on the basis of gel filtration analysis and immunological evidence to synthesise high-M_r forms of biologically inactive calcitonin [13–15]. Our results demonstrate that: the high-M_r variant of calcitonin reported by others reflects the absence, or limited activity only, of prohormone processing enzymes within the secretory pathway of these cells; the biosynthesis of calcitonin mRNA in lung carcinoma cell lines and medullary thyroid carcinoma

+ To whom correspondence should be addressed

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can involve the use of an alternative splice donor site in the first intron; the BEN cell line expresses α-CGRP mRNA; and CGRP antigenic determinants are present in BEN cell secretions, and in plasma from patients with lung carcinoma. In view of these observations, it is likely that the numerous reports in the literature describing the presence of high-M_r variants of small peptide hormones, either circulating in plasma from patients with endocrine tumours, or in secretions from established cell lines, are a reflection of aberrant post-translational processing of the precursor polyproteins.

2. MATERIALS AND METHODS

Adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol) and deoxycytidine 5′-[α-^{32}P]triphosphate (410 Ci/mmol) were obtained from New England Nuclear or Amersham International.

2.1. *Cell culture*

The BEN cell line was cultured as described by Ham et al. [14] in 50% (v/v) TC 199 Hepes-buffered medium, 50% (v/v) Dulbecco's Eagles' bicarbonate-buffered medium and 10% (v/v) foetal calf serum.

2.2. *Isolation of poly(A)-containing RNA*

Total RNA was isolated from confluent BEN cells by the method of Hall et al. [16] and the poly(A)-containing RNA fraction obtained by affinity chromatography using oligo(dT) cellulose [17].

2.3. *RNA blotting*

Poly(A)-containing RNA was analysed under denaturing conditions by electrophoresis through 1.1% (w/v) agarose gels and blotted onto Biodyne membranes as described by Taylor et al. [18]. Nick-translation of sequence-specific cDNA hybridisation probes using [α-^{32}P]dCTP was as described by Edbrooke et al. [2].

2.4. *S_1 mapping*

S_1 mapping was performed essentially according to Rosenfeld et al. [19] using a denatured ^{32}P-labelled T_4 polynucleotide kinase-labelled DNA fragment or a single-stranded ^{32}P-labelled M13 DNA probe. The latter was prepared as described by Myers et al. [42] and purified by electrophoresis on a 6% polyacrylamide/7 M urea gel.

2.5. *cDNA cloning*

Double-stranded cDNA was synthesised from total poly(A)-containing RNA essentially as described by Gubler and Hoffman [20] except that residual hairpin loops were removed by S_1 nuclease. The double-stranded cDNA was then blunt ended using T_4 DNA polymerase, internal *Eco*RI sites were methylated and *Eco*RI linkers added and cleaved. These were then chromatographed on Ultrogel AcA 34 (1 × 0.5 cm) in the presence of *Eco*RI-cut, phosphatased λgt11 DNA, and the nucleic acid recovered by ethanol precipitation from the void volume, ligated, packaged, and plated onto *E. coli* Y1088 cells (see [21–23]). Recombinant phage containing calcitonin cDNA sequences was identified using ^{32}P-labelled nick-translated calcitonin cDNA [24], plaque purified, and the inserted calcitonin cDNA then recloned directly into M13 vectors (mp10, mp11, mp18, mp19) for DNA sequence analysis [25] and S_1 mapping.

2.6. *Radioimmunoassay*

Radioimmunoassay using rabbit antisera raised against Tyr-CGRP$_{25-37}$ amide conjugated to ovalbumin was essentially as described for the calcitonin assay [26] except that ^{125}I-labelled Tyr-CGRP$_{25-37}$-amide tracer was quantitatively recovered by precipitation using goat anti-rabbit serum (10 μl) after the addition of 1 μl pre-immune rabbit serum carrier to each 400 μl assay [2].

3. RESULTS

3.1. *Expression of the calcitonin/α-CGRP gene*

We have described elsewhere the characterisation and expression of the human calcitonin/α-CGRP gene in medullary thyroid carcinoma and two small-cell carcinoma cell lines [2,27,28]. As an extension of these results we have examined the ectopic expression of the calcitonin/α-CGRP gene in an established human lung carcinoma cell line (BEN), previously shown to secrete a high-M_r form of biologically inactive human calcitonin [13–15]. Analysis of total BEN cell poly(A)-containing

RNA by RNA blotting using previously well characterised calcitonin and CGRP hybridisation probes [2] demonstrated (fig.1, tracks i,ii) an abundance of calcitonin mRNA (0.9–1.0 kb) and very much lower levels of CGRP mRNA. A number of higher M_r RNA species were also identified. Using a CGRP cDNA probe, 4.2 and 3.4 kb species of equivalent intensity of the CGRP mRNA, and a less intensely hybridising band of 2.6 kb were clearly identifiable. A larger 5.2 kb species was also just discernible. Using the calcitonin cDNA probe, the 4.2 and 3.4 kb species were again identified, though at comparatively low levels when compared with the mature calcitonin mRNA, whilst a 2.2 kb band which did not hybridise to a CGRP cDNA probe and has previously been shown to be an intermediate in the synthesis of calcitonin mRNA from a common precursor (see [2]) was also evident. In this experiment the 2.6 kb band could not be distinguished over background using the calcitonin cDNA probe, though we have identified its presence in other experiments, using total poly(A)-containing RNA from BEN, DMS 53 and DMS 153 cell lines [32] and medullary thyroid carcinoma (see [2]; Edbrooke and Craig, unpublished). Analysis of BEN cell line total poly(A)-containing RNA on a separate filter using a probe specific to the intron separating the calcitonin and CGRP exons (fig.1, track iii) as expected showed no hybridisation to the processed mRNA species, but in common with previous data [2] hybridised to the 4.2, 3.4 and 2.6 kb RNA species, but not the 2.2 kb species. Confirmation that processed α-CGRP as opposed to β-CGRP transcripts (see [2]) were expressed in the BEN cell line was obtained by S_1 mapping using a ^{32}P-labelled *NcoI/NcoI* cDNA fragment (309 bp) obtained from a cloned partially processed calcitonin/α-CGRP cDNA transcript, described elsewhere (see fig.1; also [2]). Thus, comparison of total poly(A)-containing RNA from BEN cells and from a medullary thyroid carcinoma known to express α-CGRP mRNA (see [2]; also fig.2) demonstrated the protection of the expected 244 bp fragment after S_1 analysis, thereby confirming the presence of processed α-CGRP transcripts. Analysis of medium exposed to BEN cells for 4 days using a CGRP radioimmunoassay [2] demonstrated the presence of CGRP antigenic determinants and so confirmed that the low levels of 1.1 kb CGRP mRNA observed indeed represented translatable mRNA. Parallel analysis of plasma from 12 anaplastic lung-carcinoma patients compared with normal control plasma also showed significant levels of CGRP antigenic determinants in all but one of the samples examined (fig.3 and table 1). Normal plasma CGRP levels have been reported in the range 20–50 pmol/l [41]. Although our present assay is not sensitive in this range, our data suggest that CGRP levels can be elevated as much as 200-fold in lung carcinoma.

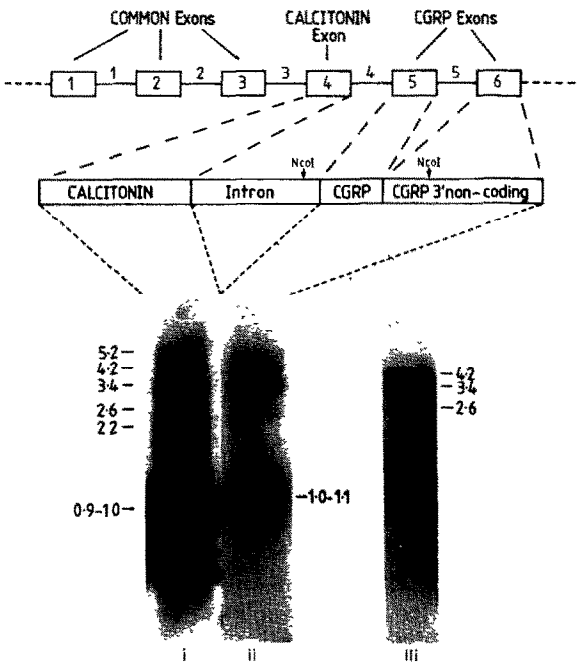


Fig.1. Expression of the calcitonin/α-CGRP gene in the BEN cell line. Schematic representation of the calcitonin/α-CGRP gene (comprising 6 exons and 5 introns) illustrates the transcribed regions of the gene from which the cDNA probes were derived (see [30]). BEN cell line poly(A)-containing RNA (7 μg) was separated in duplicate by agarose gel electrophoresis and blotted onto Biodyne filters. One filter was probed with a CGRP-specific probe (track ii; phTB58 *BglII/PstI*, see [2]). After autoradiography, the filter was washed and reprobed with a calcitonin-specific probe (track i; phTB3 *BglII/PstI*, see [2]) and autoradiographed again. The other filter was probed with an intron-specific probe (track iii; phTB58 *BglII/BglII*, see [2]). Specific activities of probes were 1–2 × 10^7 cpm/μg and all autoradiographs were for 16 h. RNA sizes are in kb.

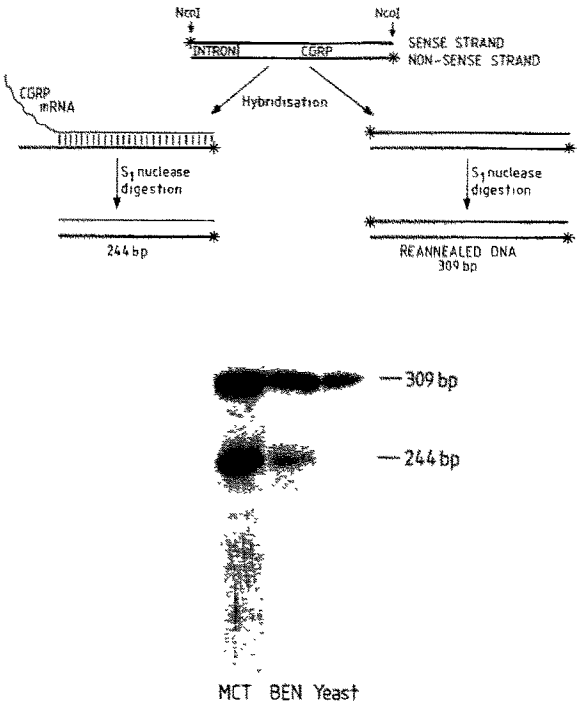


Fig.2. Identification of processed α-CGRP mRNA species. S_1 nuclease mapping of poly(A)-containing RNA from MCT (1 μg) and BEN cell (10 μg), and yeast total RNA (5 μg) using a denatured ^{32}P-polynucleotide kinase-labelled *Nco*I/*Nco*I 309 bp fragment of phTB58 (4.5×10^5 cpm/μg, see [2]).

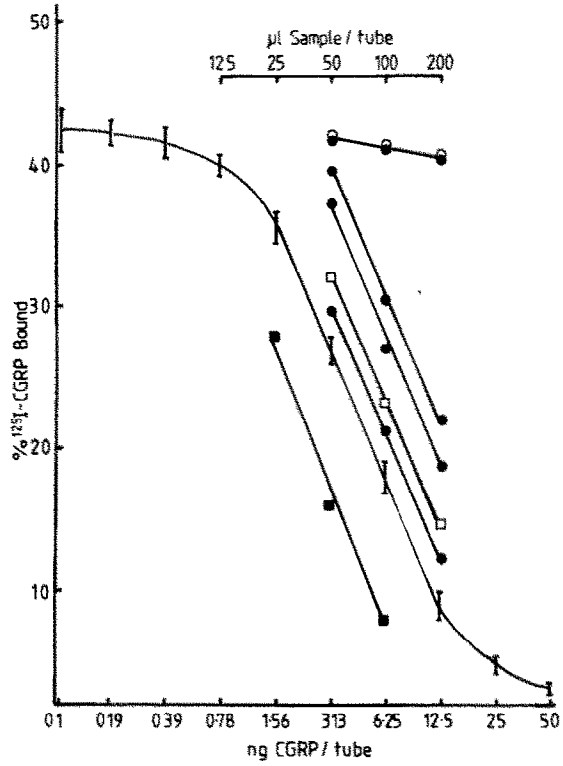


Fig.3. Radioimmunoassay of CGRP in BEN cell line 4 day secretions (□—□) and in plasma samples from patients with lung carcinoma (●—●), medullary thyroid carcinoma (■—■), and normal controls (○—○).

3.2. *The molecular basis of large calcitonin secretion*

Although the expression of the calcitonin/α-CGRP gene by the BEN cell line is identical to that of the gene in medullary thyroid carcinoma as judged by RNA blotting and S_1 mapping, such an approach will not detect single base substitutions within the genome, which may lead to amino acid substitution and so abnormal protein processing. Such lesions, the potential basis of the expression of large calcitonin, can only be identified by cDNA cloning and nucleotide sequence analysis. We have constructed a representative cDNA library (2×10^6 recombinants) in the expression vector λgt11 using total BEN cell poly(A)-containing RNA. Preliminary screening, using existing ^{32}P-labelled calcitonin and CGRP-specific cDNA hybridisation probes, showed that 0.1% of the recombinants contained calcitonin cDNA, whilst 0.01% contained CGRP cDNA sequences. Six recombinants showing positive hybridisation to calcitonin cDNA were plaque purified and the size of the inserted cDNA analysed after restriction with *Eco*RI. The largest cDNA insert (hBEN-JR2) was then restricted with *Bgl*II or *Sph*I, recloned into M13 vectors and the nucleotide sequence determined on both strands and across all internal restriction sites used for cloning. This revealed (fig.4) that the cloned cDNA fragment (723 nucleotides) comprised the entire coding sequence of the human calcitonin mRNA, and most of the 3′- and 5′-untranslated regions. The coding region was in complete agreement with previous reports of the nucleotide sequence and encodes a precalcitonin polyprotein (M_r 15451) with an amino acid sequence identical to that derived from similar analysis of clones isolated from human medullary thyroid carcinoma cDNA libraries [29,30]. Thus,

Table 1

Identification of elevated plasma CGRP in anaplastic lung carcinoma

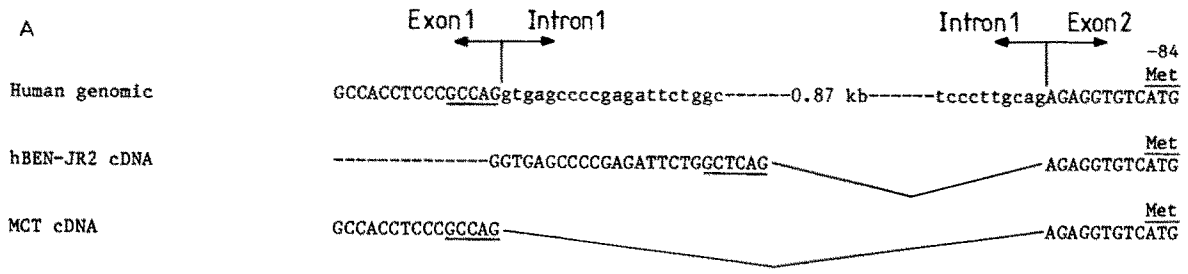
Plasma sample		CGRP (nmol/l)
Lung carcinoma	1	1.3
	2	3.4
	3	5.0
	4	11.0
	5	7.0
	6	12.0
	7	2.2
	8	5.0
	9	n.d.
	10	5.0
	11	11.0
	12	6.2
4 day BEN secretion		9.0
MCT		26
Normal plasma		n.d.
		n.d.
		n.d.

n.d., not detectable

in a cell line which secretes high-M_r forms of immunoreactive calcitonin, calcitonin(1–32) is flanked within the polyprotein by signal peptide, amino and carboxyl flanking sequence, and separated from these by basic amino acids (−2, −1 and +2, +3, +4), and the glycine residues (+1) necessary for amidation [31]. The termination codon (TAA) is also, as expected, adjacent to the asparagine residue (+25) of the flanking peptide PDN-21 [27]. Within the 3′-untranslated region no differences were observed though the cloned cDNA sequence stops short of the polyadenylation site (see [27]).

In contrast, within the 5′-untranslated region the sequence diverges from the previous published sequence at a point 10 nucleotides upstream from the initiating methionine, the known splice point between exon 1 and exon 2 of the human calcitonin gene (see [30]). The novel sequence present at the 5′-end of the BEN cell line calcitonin mRNA can also be found in the calcitonin genomic sequence within intron 1 (see fig.5A), where an alternative exon 1 splice donor site (GCTCAG) must occur 24 nucleotides to the 3′-side of the previously iden-

Fig.4. Complete nucleotide sequence of calcitonin polyprotein mRNA isolated from the BEN cell line and cloned into hBEN-JR2. Sequencing was performed on both strands, and across all restriction sites used to clone into M13 vectors. Vertical arrows indicate positions of splice junctions. Boxed sections of amino acids indicate sites for proteolytic processing or amidation (see text). The large triangle indicates the potential cleavage site for the signal peptide (see [29]).



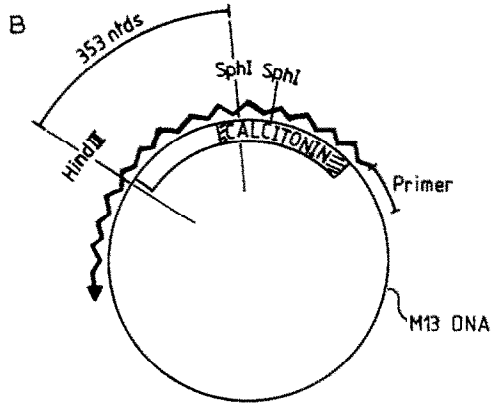
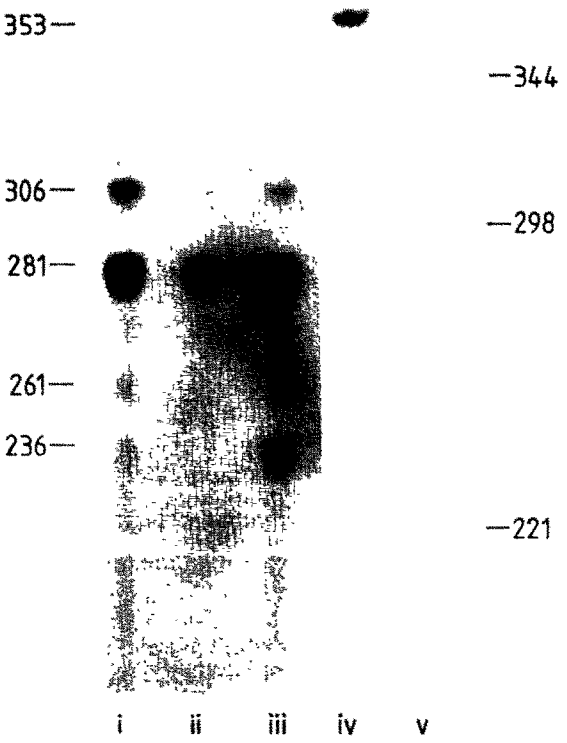


Fig.5. An alternative splice donor sequence in intron 1 of the calcitonin/α-CGRP gene gives rise to different 5′-leader sequences for human calcitonin mRNA. (A) Identification of an alternative splice donor site in intron 1. Human genomic DNA sequence is as described by Jonas et al. [30] and the 5′-leader sequence of calcitonin mRNA from MCT is as described by Le Moullec et al. [29] and Jonas et al. [30]. (B) S_1 nuclease mapping of poly(A)-containing RNA from DMS 53 (i, 100 ng), BEN cell line (ii, 200 ng), medullary thyroid carcinoma (iii, 100 ng) and *E. coli* tRNA (v, 200 ng) using a single-stranded DNA probe (353 nucleotides, track iv) generated from M13 (see section 2). ^{32}P-labelled *Hin*fI-restricted pBR322 DNA markers were electrophoresed in parallel and their relative positions indicated.



tified donor site (GCCAG), the site apparently favoured in medullary thyroid carcinoma, as judged by nucleotide sequence analysis of human calcitonin cDNA derived from this source [29,30].

To establish whether the alternative splice site is favoured, or used exclusively in the ectopic expression of calcitonin mRNA by lung carcinoma cell lines, as opposed to medullary thyroid carcinoma, we have examined by S_1 mapping the relative usage of the alternative 5′-leader sequence in total poly(A)-containing RNA isolated from BEN cells, medullary thyroid carcinoma, and the DMS 53 small-cell carcinoma cell line, the latter derived from small-cell carcinoma of the lung and known to express the calcitonin/α-CGRP gene [2,32]. S_1 mapping was performed using a 353 nucleotide ^{32}P-labelled single-stranded DNA hybridisation probe representative of the 306 nucleotide calcitonin cDNA sequence in hBEN-JR2 to the 5′ of the *Sph*I site within the calcitonin coding sequence, flanked by 47 nucleotides of M13 mp 11 polylinker and oligonucleotide primer (see fig.5B, top). Thus protection of a fragment of 306 nucleotides would reflect the use of the exon I

3′-splice site (within the intron), whilst protection of a shorter fragment of 281 nucleotides would reflect the proportion of calcitonin mRNA using the exon I 5′-splice site. As can be seen (fig.5B), in the BEN and DMS 53 lung carcinoma cell lines, and in medullary thyroid carcinoma, both splice sites are utilised, in a ratio of approx. 8:1 in favour of the 5′- as opposed to the 3′-site. Additional protected fragments at 261 and 236 nucleotides were also apparent. These are the expected size of transcripts from exons II and III of the calcitonin gene [30], with and without the 5′-leader sequence cloned in hBEN-JR2. These reflect a similar usage of the alternative splice sites in the production of the α-CGRP mRNA in the lung carcinoma cell lines and medullary thyroid carcinoma.

4. DISCUSSION

Cloning and characterisation of human calcitonin mRNA isolated from medullary thyroid carcinoma, a tumour which secretes both monomer calcitonin and high-M_r forms, has shown that, in common with most small biologically active peptides, human calcitonin is synthesised as a precursor polyprotein, subsequently cleaved from the polyprotein by proteolytic enzymes within the secretory pathway of thyroidal C-cells [27,28]. These enzymes recognise basic amino acids flanking the amino- and carboxyl-terminal ends of the calcitonin peptide. The carboxyl-terminal proline (+32) is amidated (a requirement for biological activity) by an amidation enzyme which utilizes the amino group of glycine (+1) in the polyprotein as the source of an amide [31]. Studies on the BEN cell line have previously shown that these cells secrete an immunoreactive calcitonin which is biologically inactive, and of predominantly high M_r (M_r 7000–40000, see [13–15]). Studies using human and rat calcitonin cDNA hybridisation probes have in addition shown the presence, by RNA blotting, of an abundant BEN cell calcitonin mRNA [33,34] which on the basis of cell-free protein synthesis and immunoprecipitation directs the synthesis of a 17 kDa protein, similar to the mRNA-directed immunoprecipitable calcitonin polyprotein identified using poly(A)-containing RNA isolated from human medullary thyroid carcinoma [34].

Our studies described above now define in molecular terms the basis of secretion by the BEN cells of high-M_r forms of calcitonin. The transcription and post-transcriptional processing of the human calcitonin/α-CGRP gene by the BEN cell line are identical in every way, as judged by RNA blotting and S_1 mapping, to calcitonin gene expression in medullary thyroid carcinoma [2]. Calcitonin mRNA derived from a medullary thyroid carcinoma known to secrete calcitonin monomer [26,27] and calcitonin mRNA from the BEN cell line showed no differences within the coding region of the mRNA. Thus, the ectopic secretion of larger forms of immunoreactive calcitonin by the BEN and other lung carcinoma cell lines is not a reflection of abnormal mRNA processing or point mutations in the reading frame of the mRNA corresponding to the sites in the polyprotein required for post-translational processing. The inability of lung cells to secrete calcitonin monomer reflects inefficient proteolytic cleavage of the polyprotein prior to secretion. Studies on mRNA-directed protein synthesis using *Xenopus* oocytes would support this view, since proteolytic processing of proinsulin is not an obligatory requirement of the secretory process [35]. Consequently, the ectopic secretion of high-M_r calcitonin, and in all probability high-M_r forms of other peptides such as ACTH secreted ectopically by endocrine tumours (see [36]), reflects the absence of expression, or the limited expression of proteolytic processing enzymes, within the secretory pathway of tumour cells. The unexpectedly high M_r as judged by gel filtration of some of the large calcitonin secreted by the BEN cell line (M_r 40000) may reflect molecular asymmetry or glycosylation, both of which can lead to disparities in M_r estimations. Since glycosylation of the rat calcitonin polyprotein has been demonstrated [37,38], it is likely that glycosylation in addition to aberrant proteolytic processing may well contribute to the heterogeneous nature of large calcitonins secreted by the BEN cell line, other cell lines and tumours.

The identification of significant levels of immunoreactive CGRP in BEN cell secretions and in the plasma of 11 out of 12 anaplastic lung carcinoma patients examined adds weight to accumulating evidence [2] that CGRP may be a valuable diagnostic marker peptide for lung car-

cinoma in addition to medullary thyroid carcinoma (see [2,8]). In medullary thyroid carcinoma, CGRP measurements are valuable adjuncts to calcitonin measurements [2,8]. However, the frequency of elevated plasma CGRP in anaplastic lung carcinoma (11 out of the 12 samples measured) suggests that CGRP will be an important diagnostic marker for this disease.

The use of alternative splice donor sites, leading to different 5′-leader sequences for human calcitonin mRNA at similar frequencies in lung carcinoma cell lines and medullary thyroid carcinoma, has also been reported in studies on the rat calcitonin/α-CGRP gene [40]. Both reflect the favoured use of one of two alternate splice sites, which, unlike in a human β-thalassaemia where the coding sequence is affected [39], have no obvious deleterious effect on gene expression. The alternative use of these splice sites, which appears to be a phenomenon common to all calcitonin- and α-CGRP-producing cells, should not, however, be confused with other post-transcriptional processing events involving cleavage and polyadenylation. The latter, which determine in a tissue- or cell-specific manner whether calcitonin or α-CGRP are the major products of the calcitonin/α-CGRP gene, are modulated via a transacting factor (see [2,40]).

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