Expression of the Gastrin-releasing Peptide Gene in Human Small Cell Lung Cancer

EVIDENCE FOR ALTERNATIVE PROCESSING RESULTING IN THREE DISTINCT mRNAs*

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cDNA clones to human prepro-gastrin-releasing peptide (prepro-GRP) mRNA detect synthesis of prepro-GRP-related transcripts in 4 of 7 small cell lung cancer (SCLC) cell lines and 1 of 2 metastatic SCLC tumors examined. A correlation is noted between prepro-GRP gene expression and the occurrence of bombesin-related immunoreactivity in SCLC cell lines. Examination of the structure of prepro-GRP transcripts found in SCLC reveals three types of prepro-GRP mRNA which differ in the structure of a putative GRP-associated peptide in the pro-GRP precursor. The subcellular distribution of prepro-GRP-related RNAs and structure of SCLC-derived prepro-GRP cDNA clones suggest that all three types of transcript could function as mRNAs, although there are differences in the prevalence of the different RNA types in different cellular compartments. Comparison of the sequence of cDNA clones with the sequence of a genomic prepro-GRP clone reveals that the three forms of prepro-GRP mRNA arise from a single primary transcript which undergoes alternative processing from two splice donor sites to two splice acceptor sites. The predicted amino acid sequence of the translated products of the three mRNAs are quite distinct, leading to predicted pro-GRP molecules of differing structure.

The cell in the normal bronchial epithelium to which small cell lung carcinoma (SCLC) cells bear the greatest ultrastructural and biochemical resemblance is the Kulchitsky or pulmonary endocrine cell (Bensch et al., 1965). The exact function of the cells is unknown, but they are prominently found in neonatal and injured lungs, and may function in control of regional ventilation and perfusion (Gould et al., 1983). The normal pulmonary endocrine cell has many properties of neuroectodermal "APUD" cells including elaboration of peptide hormones and biogenic amines. Peptide hormones for which there is good evidence of elaboration by SCLC cells in vivo or in vitro include vasopressin, pro-opiomelanocortin, calcitonin, and bombesin-like peptides (reviewed in Gazdar and Carney, 1984).

Bombesin is a tetradecapeptide in amphibians, whose mammalian homolog is thought to be gastrin-releasing peptide (GRP), originally isolated from porcine non-antral gastric tissue. The carboxyl-terminal 14 amino acids of mammalian gastrin-releasing peptide are very similar to amphibian bombesin (McDonald et al., 1979), especially the carboxyl-terminal heptapeptide (see Fig. 1). Bombesin-like immunoreactivity has been found in brain (Brown et al., 1978; Ghaté et al., 1984) as well as in the gut (Walsh et al., 1979). Bombesin-like immunoreactivity has been also detected in pulmonary endocrine cells in fetal bronchial epithelium (Wharton et al., 1978), human pulmonary carcinoids (Yang et al., 1983; Tamai et al., 1983), and human SCLC or SCLC-derived cell lines (Erisman et al., 1983; Moody et al., 1981; Sorenson et al., 1982). Using RNA from a pulmonary carcinoid, Spindel et al. (1984) obtained cDNA clones for the prepro-GRP gene, which encode a putative pro-GRP molecule consisting of a signal sequence (amino acids -23 to -1), GRP (amino acids 1-27), and a GRP-associated peptide (amino acids 31-125) of unknown function (see Fig. 1). Recently Spindel et al. (1986) have found two types of prepro-GRP cDNA clones in a cDNA library from pulmonary carcinoid tissue, which differ in the presence of a 19-base insertion in the 3’ GRP-associated peptide occurring after amino acid 98 in pro-GRP. This alteration would provide for at least two forms of pro-GRP, differing in the carboxyl-terminal GRP-associated peptide.

In view of the complexity of prepro-GRP transcription implied by the existence of two types of human cDNAs, and in view of the recently observed role of GRP as a potential mitogen for SCLC lines (Weber et al., 1985; Cuttitta et al., 1985), we sought to characterize prepro-GRP gene expression in lung cancer cell lines. Our results indicate a marked correlation between prepro-GRP gene expression and the occurrence of bombesin-like immunoreactivity. We observe steady-state synthesis of both forms of mRNA implied by the clones obtained previously by Spindel et al. (1986). We also define a third form of prepro-GRP mRNA and show that all three forms of prepro-GRP mRNA can arise by alternative processing from a single primary transcript.

MATERIALS AND METHODS

Cell Lines and Cell Culture—The origin and culture of the cell lines described in this paper have been described in detail (Carney et al., 1983; Tamai et al., 1983; Sorenson et al., 1982).
Cell lines NCI-H209, NCI-H128, NCI-H345, NCI-H378, NCI-H249, and NCI-H524 all derive from patients with typical SCLC, and NCI-H510 derives from an extrapulmonary small cell-like tumor. The non-small cell lung cancer and other cell lines used here are all maintained in RPMI 1640 medium plus 10% fetal bovine serum. Tumors obtained at autopsy were frozen at −70°C until processing.

Nucleic Acids—DNA was extracted as described by Polsky et al. (1978) and RNA as described by Chirgwin et al. (1979), with poly(A*) RNA selection as originally described by Aviv and Leder (1972). Total cytoplasmic RNA was prepared after swelling 0.5–1 × 10^6 SCLC cells in 0.01 M Tris, pH 7.5, 0.01 M KCl, 0.0015 M MgCl₂ for 5 min at 4°C, followed by lysis with eight to 16 strokes of a Dounce homogenizer, monitored by phase contrast microscopy. Lysates were centrifuged at 1000 rpm for 8 min in a Sorvall GLC-4 centrifuge. The supernatant was removed, and the loose nuclear pellet was lysed with guanidine thiocyanate while the supernatant was also further processed by mixing with 7 volumes of 4 M guanidine thiocyanate, 0.25 M Na citrate, pH 7.0, 0.01 M 2-mercaptoethanol (crude cytoplasmic RNA). Alternatively the cytoplasm was centrifuged through 10–25% sucrose gradients as described by Huang and Grollman (1972), and the mono- and polyribosome fractions were determined by UV absorbance. Appropriate fractions were pooled, precipitated with ethanol, extracted with phenol, and precipitated with ethanol (ribosome associated RNA).

GRP plasmids pB1 and pB12 were as described by Spindel et al. (1984) (see Fig. 1). The plasmid pB19 was also used, which is identical to pB12 except for the presence of a 19-base insertion in the 3′ portion of the gene. The sequence presented in Spindel et al. (1984) contains the 19-base insertion present in pB12, at positions 419-437, whereas pB12 lacks this insertion (Spindel et al., 1986). The 19-base insertion does not alter the coding sequence for GRP, but affects the carboxyl-terminal GRP-associated peptide in the pro-GRP precursor molecule.

DNA and RNA were transferred to nitrocellulose (Southern, 1975) after electrophoresis through non-denaturing or formaldehyde-containing gels, respectively. Hybridization to nitrocellulose blots proceeded as described by Wahl et al. (1979) using probes prepared by nick translation (Rigby et al., 1977) of DNA fragments purified by gel electrophoresis.

S1 Nuclease Protection—S1 nuclease protection assays of labeled DNA probes hybridized to RNA were conducted as described by Battey et al. (1983). The probes used were mass-labeled with [³²P]dATP by primer extension of appropriate subclones in M13, followed by preparative gel electrophoresis and electroelution to obtain single strand probes, covalently linked to primer and polylinker sequences. S1 nuclease protection assays were also performed after hybridization to genomic DNA. Fifteen µg of SCLC DNA or salmon sperm DNA were cleaved to completion with, in our case, PstI. The digested genomic DNA was mixed with a [³²P]-labeled single-stranded probe prepared as described above using an M13 subclone. The mixed probe and DNA were denatured by boiling for 2 min and then hybridized for 36 h at 45°C in 70% formamide, 0.4 M NaCl, 0.02 M Tris, pH 7.5, and 0.002 M EDTA. Hybridization reactions were diluted to 400 µl, adjusted to 0.3 M NaCl, 0.05 M Na acetate, pH 4.5, and 0.003 M ZnSO₄, and then 2500 units of S1 nuclease (Boehringer Mannheim) was added. Digestion proceeded at 37°C for 2 h and was terminated with phenol/chloroform extraction and ethanol precipitation, followed by electrophoresis on a 5% polyacrylamide sequencing gel. The gel was then transferred to 3MM paper, dried, and exposed to XAR-5 film for 12–48 h.

cDNA Synthesis and Genomic Clones—A cDNA library to poly(A*) RNA from SCLC line NCI-H209 was constructed essentially as described by Gubler and Hoffman (1983), except that after synthesis of the second strand, the cDNA was methylated, and EcoRI linkers were added and ligated into the EcoRI site of Agt11 (Young and Davis, 1983) as described by Littman et al., 1985. Nucleotide sequences of selected subclones in M13 were obtained by the dideoxynucleotide chain termination technique (Sanger et al., 1977) as described in a Bethesda Research Laboratory Manual (1981, Gaithersburg, MD).

A genomic library constructed from partially MB01-digested fetal liver DNA (Ravetch et al., 1981) cloned into the BamHI site of C132B11 as described by Chen et al. (1977) was screened for clones containing prepro-GRP sequences. Subclones for sequencing were initially subcloned in pBR322 and then prepared in M13.

**RESULTS**

**Fig. 1. Comparison of bombesin and GRP.** The amino acid sequences of bombesin and GRP are compared and the determinant of monoclonal antibody 2A11 to bombesin is indicated. The overlap of prepro-GRP cDNA clones pB1 and pB12, as well as restriction enzyme sites used to generate probes A and B used in Figs. 2, 3, 5, and 6, are shown. Restriction enzyme recognition sites are: Sall, SstI, PstI, PvuII.

**Fig. 2. Expression of prepro-GRP in SCLC cell lines.** RNA from the indicated sources was electrophoresed (10–15 µg of total (T) and 2 µg of poly(A*) (P) RNA) and transferred to nitrocellulose. Hybridization to probe B from Fig. 1 is shown. The following amounts of bombesin-like immunoreactivity were detected in these cell lines by radioimmunoassay with monoclonal antibody 2A11 (in pmol/mg protein): NCI-H209, 17.4; NCI-H128, 18.4; NCI-H345, 7.8; NCI-H510, 5.8; NCI-H524, 3.8; NCI-A549, <0.01; NCI-H23, <0.01; NCI-H125, <0.01; NCI-157, <0.01; NCI-BL1, <0.01; NCI-234, <0.01. Lym and Mel, lymphoblastoid and melanoma cell lines, respectively.
B also detects species of approximately 7.8 and 3.8 kb. The larger of these two species is clearly diminished when cytoplasmic RNA is examined (data not shown), consistent with its role as a nuclear precursor of mature prepro-GRP mRNA. NCI-H378 is a SCLC cell line which does not contain significant quantities of prepro-GRP mRNA. Adenocarcinoma-derived cell lines A549, NCI-H23, HCl-H125 as well as the large cell undifferentiated carcinoma cell line NCI-H157 also show no evidence of prepro-GRP mRNA expression. As expected, melanoma cell line NCI-H234 and the human lymphoblastoid cell line NCI-H128 BL1 also show no prepro-GRP transcripts. The legend to Fig. 2 lists the amount of bombesin-like immunoreactivity found when these cell lines were examined by a radioimmunoassay using monoclonal antibody 2A11 (Cuttitta et al., 1985, see Fig. 1) or a heteroantiserum raised to bombesin (Moody et al., 1983; Carney et al., 1985). There is a correlation between the occurrence of prepro-GRP mRNA and bombesin-like immunoreactivity in the cell lines examined here.

Fig. 3 shows that probe A from the 5' SacI-PvuII fragment from pB1 (see Fig. 1), when labeled by primer-extension in an M13 subclone, is protected by total RNA from NCI-H510 and H209 to yield a single species, the expected 195-base fragment which corresponds to a portion of the prepro-GRP signal sequence, the entire GRP moiety, as well as a portion of the carboxyl GRP-associated peptide. This probe also detects an analogous species in RNA derived from one of two liver metastases obtained at autopsy, each from a different patient with SCLC. An axillary node metastasis from a patient with large-cell lung cancer contains no RNA protecting the probe. NCI-H524 and NCI-H249, SCLC cell lines amplified for the c-myc and N-myc proto-oncogenes, respectively (Gazdar et al., 1985; Nau et al., 1986), show no evidence of prepro-GRP mRNA by this assay. This result again correlates with the radioimmunoassay for bombesin-like peptides (see Fig. 3 legend).

We conclude from Figs. 2 and 3 that GRP is a major source of bombesin-like immunoreactivity in the SCLC cell lines examined to date. No example has yet been encountered of a cell line deriving from a non-small cell histologic type of lung cancer associated with expression of prepro-GRP mRNA. There are, however, examples of SCLC-derived cell lines which do not contain detectable prepro-GRP transcripts. In experiments not shown here, no obvious amplification or rearrangement of the prepro-GRP gene was detected in DNAs from several SCLC and non-SCLC cell lines.

**Nature of Prepro-GRP Transcripts in SCLC**—To approach the question of whether SCLC cells in vitro actually synthesize multiple forms of prepro-GRP mRNA, a cDNA library constructed to poly(A)+ RNA from SCLC cell line NCI-H209 was screened with probe B (shown in Fig. 1). Twelve prepro-GRP cDNA clones were characterized, and these were of the types shown in Fig. 4. Type I prepro-GRP clones (of which seven were obtained) have the same structure as the insert (+) clone of Spindel et al. (1986) with the 19-base insertion found here after position 363 in the Type I clone described in Fig. 4. Type III prepro-GRP cDNA clones, of which there were four obtained here and which correspond to the insert (−) clone of Spindel et al. (1986), lack the 19 bases after position 363. In addition, the SCLC-derived cDNA library contained one clone of the Type II (Fig. 4). The Type II clone has the 19 bases present in Type I but deleted in Type III clones, and instead has a deletion of 21 bases immediately 3′ (positions 383–403) of the bases deleted in the Type III clone.

Fig. 4 also demonstrates that the sequence of a prepro-GRP genomic DNA clone accounts for the observed structure of the cDNAs by use of alternative processing pathways. It can be seen that the 19 base pairs present (363–382) in Type I cDNA are found in genomic DNA between two potential splice donor sites, with both donor sites followed immediately by a break in homology with Type III (5′ donor) or Types I and II (3′ donor) cDNA clones. The sequence after both potential splice donor sites suggests the beginning of an intron (Breathnach and Chambon, 1981) (note underlined GT dinucleotides in Fig. 4). The 21 bases found in Types I and III (383–403) but deleted in Type II cDNA clones appear with resumption of homology to Types I and III cDNA between two splice acceptor consensus sites in genomic DNA (Mount, 1982; note underlined AG dinucleotides in Fig. 4) located approximately 4.4 kb 3′ of the start of the intron. Resumption of homology with the Type II cDNA clone occurs after the more 3′ splice acceptor site.

The most straightforward interpretation of these data is that two potential splice donors and two potential splice acceptors are used in this gene. Type I cDNA clones arise from RNA that reflects splicing from the more 3′ splice donor (at the 3′ end of the 19 bases deleted in Type III clones) to the more 5′ splice acceptor (at the 5′ end of the 21 bases deleted in the Type II cDNA clone), resulting in the inclusion of both the 19 bases between the two splice donor sites and
Fig. 4. Sequence of cDNA and genomic prepro-GRP clones. A schematic representation of the pre-GRP putative precursor molecule in shown in the center of the panel, including the signal sequence (cross-hatch right to left), GRP, and the carboxyl GRP-associated peptide (cross-hatch left to right). The three types of cDNA clones are indicated as Types I, II, and III in the nucleotide sequence shown below this, and below the nucleotide sequences are the inferred amino acid sequences of this region of pre-GRP. The sequence coordinates are with respect to the initiator codon of pre-GRP in the cDNA clone described by Spindel et al. (1984). The sequence of this region of the Type I prepro-GRP cDNA clone obtained from H209 is identical to that of Spindel et al. (1984) except that a C was found here at position 401 in both Type I and Type III cDNA clones from NCI-H209. Above the representation of pre-GRP is the sequence of a portion of a prepro-GRP genomic clone in the region of the proposed splice junctions to account for the observed types of cDNA clones. The 19 bases between two putative splice donor sites are indicated by 19 and 21 sites found between two putative splice acceptor sites. The distance between donor and acceptor regions is indicated to be approximately 4.4 kb, as determined by restriction mapping of the genomic clone. The 19 and 21 bases are both present in Type I and deleted in Types III and II cDNA clones, respectively. The splicing patterns which would give rise to mRNAs accounting for each of the three cDNAs is indicated by I and II above and III below the sequence obtained from the genomic clone. The upper case letters in the genomic sequence correspond to exons, and lower case letters to the sequence which would be in the intron irrespective of the splicing pattern used.

the 21 bases between the two acceptor sites in the mature Type I cDNA clone. Type II cDNA clones arise from RNA which use the same splice donor site as Type I clones, but splicing occurs to the more 3’ acceptor site, therefore deleting the 21 bases between the acceptor sites from mature RNA. Type III cDNA clones arise from mRNA which has utilized the more 5’ splice donor site and the more 5’ splice acceptor site, resulting in deletion of the bases between the two donor sites and retention of the bases between the two acceptor sites. These relationships are shown diagrammatically in Fig. 4. It should be noted that cDNA clones of all three types can be demonstrated to have a polydeoxyadenosine sequence at their 3’ ends (data not shown), confirming that they arise from potential mRNA species.

Genomic Representation of Prepro-GRP cDNA Clones—An alternative explanation for the multiple types of prepro-GRP cDNA clones obtained might be multiple GRP loci or the existence of allelic prepro-GRP genes. Fig. 5, panel I, shows that probe C, a PvuII-PvuII fragment from the 3’ GRP-associated peptide region of pB19, identical to the Type I clones derived here, hybridizes to 4.6- and 2.9-kb genomic HindIII fragments, whereas a 5’ PstI-AccI fragment from pB19 extending to just 10 bp beyond the 19 bases present between the two putative splice donor sites in genomic DNA (as depicted in Fig. 4) hybridizes only to the 4.6-kb fragment (probe D, Fig. 5). A 3’ AccI-PstI fragment from pB19 (probe E, Fig. 5) hybridizes to the 2.9-kb fragment alone. Analogous hybridizations with probe B (Fig. 1) representing the corresponding PvuII-PvuII fragment from pB12, identical to the Type III clones obtained here, detect the same 4.6 and 2.9 HindIII genomic fragments, whereas probe A from pB1 (see Fig. 1) detects 11- and 4.6-kb genomic fragments (data not shown). Thus, the putative exon/intron boundary observed in the fetal liver-derived genomic clone is also present in genomic DNA from NCI-H209, the SCLC cell line from which the cDNA clones were derived.

To examine whether two alleles of the prepro-GRP gene could exist, different by their arrangement of the 19 base pairs between the two potential splice-donor sites, genomic DNA from SCLC line NCI-H209 was digested with PvuII and hybridized to probe C which had been prepared from a primer-extended M13 subclone of the PvuII-PvuII fragment of pB19 (identical to Type I cDNA clones obtained here). The hybrids were then digested with nuclease S1. Fig. 5, panel 2, shows that genomic DNA from NCI-H209 specifically protects from 51 nucleotide fragments of 170 and 220 bases. This result is consistent with the structure shown in Fig. 4, where the 19 bases between the splice donor sites are 5’ to the intron boundary utilized by the Type I cDNA clone, contiguous with the 5’ 150 bases of the Type I probe (see diagram, Fig. 5). If an allele existed with noncontiguity of the 19 bases, then a protected fragment of 150 bases after S1 digestion would also be expected. This, however, is not observed. The 220-base fragment in Fig. 5, panel 2, reflects protection of the entire probe resulting from hybridization to two separate PvuII genomic DNA fragments 5’ and 3’ of the intron. The 220-base protected fragment is more intense in Fig. 5 than the 170-base species probably because S1 nuclease efficiently digests the probe across from the short gap which exists between hybridizing regions of the separate PvuII genomic fragments. The results presented in Fig. 5 are therefore consistent with a single prepro-GRP allele in a single prepro-GRP locus as responsible for all types of cDNA obtained previously.

Prevalence of Prepro-GRP Transcripts—To examine whether other types of prepro-GRP transcripts exist, a single \(^{32}P\)-labeled probe complementary to mRNA was prepared after subcloning in M13 and primer extension of the PuuII-PuuII segment of a Type I cDNA clone. An analogous probe prepared from pB12, identical to a Type III cDNA clone, was also prepared. Fig. 6 (lanes 1-7) shows that the Type I probe is protected by RNA from NCI-H209. Residual, undigested single-stranded probe linked to vector and primer sequences is present at 288 bp. Protected species include one at 220 bp, which would be expected if transcripts corresponding to the full length of coding sequences in the probe were present. Type I probe hybridizing to Type III mRNA would form a 19-base loop at the inserted sequences, which would be susceptible to digestion by S1 nuclease (see schematic diagram below Fig. 6). This would divide the protected region of the probe into a 150-base and approximately 50-base segment. In addition to protection of the 220-base full-length species, Fig. 6 shows that total RNA, cytoplasmic RNA, nuclear RNA, and poly(A\(^+\)) from NCI-H209 protect fragments from the Type I probe of approximately 150 and 40-60 bp. A 170-base fragment is also protected by poly(A\(^+\)) and nuclear RNA and is present but less abundant in fragments protected by cytoplasmic, ribosome-associated RNA. We interpret these data as being evidence for the presence of Type III mRNA (accounting for the 150- and 40-60-base segments), Type II mRNA (accounting for the 170-base species), as well as Type I mRNA.

The analogous probe prepared from a Type III cDNA clone is also protected by total, nuclear, cytoplasmic, and poly(A\(^+\)) RNA from NCI-H209 (Fig. 6, lanes 8-14). Undigested, full-length probe including M13 polylinker and primer sequence is present at 269 bp, and prepro-GRP-derived transcripts account for the observed species at 200 bases. Comparison with the structures of the cDNA clones presented in Fig. 4 will reveal that the 150-base species can be accounted for as arising from either Type I or Type II mRNA hybridized to Type III probe, with the label in the Type II mRNA/Type III probe hybrid expected to be more sensitive to S1 nuclease than the Type I mRNA/Type III probe hybrid. Thus, all species protected from S1 digestion by RNA in Fig. 6 can easily be explained by the gene structure and clones shown in Fig. 4 and provide further evidence that the multiple types of prepro-GRP cDNAs are not cloning artifacts, but accurately represent mature mRNAs present in the cytoplasm of GRP-producing cells. There is no evidence in these experiments for any additional RNA species.

DISCUSSION

The results presented here confirm that the prepro-GRP gene is a major source of bombesin-like immunoreactivity in human SCLC cells. Additionally, these experiments show that...
SCLC cells growing in vitro produce at least three different potential prepro-GRP RNAs. Their structural differences arise after processing which occurs in relation to splice junctions in genomic DNA. The structure of cDNAs to these transcripts suggest alternative splicing, involving at least two donor and two acceptor sites.

Bombesin-like immunoreactivity had been demonstrated in human SCLC by Moody et al. (1981, 1983), Erisman et al. (1982), Sorensen et al. (1982), and Wood et al. (1981). However, the source of this immunoreactivity has remained confusing. Mammalian gastrin-releasing peptide had previously been shown to be a 27-amino acid peptide with striking homology in its carboxyl-terminal portion to amphibian bombesin (McDonald et al., 1979). Yamaguchi et al. (1983) had suggested that GRP, as opposed to bombesin, was expressed in SCLC. Yet examination of bombesin-like immunoreactivity in SCLC revealed heterogeneity in the material detected by anti-GRP or anti-bombesin antisera. Peptides consistent with full-length GRP, as well as shorter peptides, were observed by some workers (Yamaguchi et al., 1983), whereas other antisera recognized material that co-eluted with amphibian bombesin (Moody et al., 1983). A number of additional bombesin-like peptides are known in amphibians including ranatensin and alytensin (Erisamer and Melchiorri, 1973). Minamino et al. (1983) subsequently described neuromedin B in porcine spinal cord, differing from the carboxyl-terminal sequence of GRP in only 3 positions. The independent demonstration of RNA hybridizing to 5' and 3' prepro-GRP derived sequences (Figs. 3 and 2, respectively) conclusively proves that it is the prepro-GRP gene expressed in a number of the SCLC lines described here. These experiments therefore support the contention of Bostwick et al. (1984) who used antisera to the GRP 1–16 fragment to suggest that GRP is a major bombesin-like peptide in human SCLC. Whether other bombesin-related peptides are expressed in cell lines not having RNA which hybridizes to prepro-GRP derived probes is a subject for further study.

Of 9 SCLC cell lines or tumors examined here, 5 contained large amounts of bombesin-like immunoreactivity and had evidence of prepro-GRP gene expression by either nitrocellulose filter hybridization or S1 nuclease protection. The SCLC cell lines negative for prepro-GRP gene expression include NCI-H524 and NCI-H249, amplified for c-myc and N-myc, respectively. Amplification of c-myc has previously been associated with decreased bombesin-like immunoreactivity in SCLC cell lines (Gazdar et al., 1985). A correlation also suggested here is the absence of prepro-GRP gene expression in lung cancer cell lines of non-small cell histologic type. This suggests the utility of prepro-GRP gene expression as a marker for neuroendocrine differentiation in lung cancers.

Our experiments also describe several notable features of prepro-GRP gene structure and its expression in SCLC cells. Three forms of prepro-GRP mRNA are produced by SCLC in vitro. The genomic DNA sequence presented in Fig. 4 offers a clear explanation for the multiple forms of prepro-GRP mRNA observed here. There is evidence for two splice donor sites separated by an intron from two splice acceptor sites. It is of interest that Type I (3' splice donor to 5' splice acceptor) and Type III (5' splice donor to 5' splice acceptor) splicing patterns lead to more abundant transcripts than the Type II (3' splice donor to 3' splice acceptor) patterns, and we have not yet detected use of the more 5' splice donor to the more 3' splice acceptor in any clone thus far examined. It is intriguing to speculate that the steady-state level of these differing transcripts may reflect the relative activity of different splicing mechanisms.

The generation of protein diversity by alternative use of a splice donor site to a downstream exon has been documented in the case of immunoglobulins (Early et al., 1980), and alternate acceptors have been shown in the case of fibronectin (Schwarzbauer et al., 1983), calcitonin (Amara et al., 1982), and pro-opiomelanocortin (Oates and Herbert, 1984). The prepro-GRP system is distinguished by its use of both alternate donor and acceptor sites in processing transcripts found in SCLC cells growing in vitro. Fig. 6 shows that there appears to be a greater abundance of the RNA species protecting the 170-base fragment of the Type I probe in the nucleus as opposed to cytoplasmic compartments. This 170-base fragment could be protected by mature Type II mRNA, as suggested previously, but could also be protected by intermediates in the splicing pathway leading to mature Type I and II mRNAs (Konarska et al., 1985), as cleavage of their common precursor would be expected to occur after the more 3' splice donor. While the abundance of the 170-base fragment protected by nuclear RNA raises the possibility that these intermediates may exist in relatively high concentrations in the nucleus, the existence of the 170-base protected fragment by poly(A) RNA as well as by RNA associated with ribosomes is evidence that it also reflects a mature, potentially functional mRNA.

It is currently unclear what may be the role of the three different putative carboxyl GRP-associated peptides potentially derived from the cDNA clones found here. It is possible that the various forms of prepro-GRP mRNA are all equally functional as far as GRP production is concerned, and that the existence of different forms of mRNA in the 3' prepro-GRP region is the result of an ambiguous or error-prone splicing mechanism. It is also possible that the different forms of the GRP-associated peptide may have individual physiologic or developmental significance, or perhaps affect the processing of the GRP prohormone. Further studies can utilize the structures determined here to provide reagents to address these issues. In addition, SCLC may contain determinants critical to the use of one splicing pathway over another, and thus allow delineation of factors used in the regulation of differential RNA splicing.

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