crossed to an uninjected male. Nine of 15 progeny from three litters thus far tested have inherited the pIf-derived sequences. The numbers of offspring with these sequences in each litter were six of six, zero of four, and three of five. The second litter was killed at birth and therefore the sex of the litter members was not examined; however, the sex ratios of the first and third litters were normal (three males out of six, and two males out of five). That all of the first six mice showed the sequence and all of the next four did not was unexpected, but is best explained as a statistical anomaly. The ontogenic history of the mouse primordial germ cell is such that randomization of this cell population occurs prior to entry into genital ridge. At present, there is no evidence that suggests a special relation between oocytes ovulated during any particular estrous cycle.

Consistent with the notion that the sequences were inherited is the observation that the restriction patterns of the DNA from the offspring were indistinguishable from those of the parent. Digestion with Bam HI, Pvu II, or Xba I gave identical patterns in parent and offspring (Fig. 3, a to c). Particularly persuasive is the digest with Xba I; a partial digest of one of the offspring's DNA and of mouse If-4 gave the same multiple bands (Fig. 3c). This result shows that not only are the closest Xba I sites in parent and offspring located at similar distances from the plasmid sequences, but more distant sites are also similarly or identically spaced. Subsequent complete Xba I digests of If-4 and offspring No. 2 resulted in a single band of the same size as the other five offspring (data not shown). These results provide evidence that the pIf sequences were integrated into a host chromosome.

The introduction of foreign DNA in a mouse chromosome without disruption of the meiotic process presents the possibility of producing large colonies of mice carrying transferred sequences. This capability is essential for many kinds of studies of gene transfer into mice. The production of such a colony, however, requires that the transferred material remain stable in the genome over several generations. We tested the stability of the pIf-derived sequences in the If-4 line by breeding one of its offspring to an uninjected male mouse to produce F_2 progeny. Whole animals were killed; and their DNA was extracted, digested with Pvu II, and subjected to filter hybridization with pIf as the probe. Two of the first eight offspring produced by one of the F_1 mice showed clear homology to the probe, with a restriction pattern indistinguishable from the F_1 parent or from the original transformed mouse, If-4 (Fig. 3d). This second generation of germ line transmission constitutes evidence for the stability of the transferred material.

The integration of plasmid sequences and their transmission to offspring means, for example, that mice can be backcrossed to produce homozygotes for the transferred sequences, making possible the study of crossover events within a DNA segment whose sequence is well defined, and facilitating mapping studies by both Mendelian and somatic cell genetic approaches. Sequences present in small organs can be studied by pooling tissue from many animals. Breeding tests can also be used to determine whether genes transferred into mice are integrated randomly or reproducibly into a specific site. This issue is of importance if attempts at gene replacement are to be made.

Our data, as well as those from several other laboratories, indicate promise for the technique of pronuclear injection for studying gene action during mammalian development. Our initial report that such injections could succeed in transferring genes into developing mice has been confirmed (*11–14*). The successful transfer of human insulin into fetal mice by pronuclear injection has been demonstrated (*11*); and subsequently, the retention of the human β-globin gene in the DNA of fetal mice was described (*12*). Supporting evidence for germ line transmission of transferred genes has also been gathered (*13, 14*), and expression of genes injected into the pronucleus has been observed at late fetal stages and in adult mice (*12, 14*).

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15. Supported by NIH grants GMO9966 (to F.H.R.) and GMO7959-01 (to J.W.G.).

30 September 1981; revised 30 October 1981

High Levels of Intracellular Bombesin Characterize Human Small-Cell Lung Carcinoma

Abstract. *"Small cells" or "oat cells" characterize a virulent form of lung cancer and share many biochemical properties with peptide-secreting neurones. The neuropeptide bombesin is present in all small-cell lines examined, but not in other lung cancer cell lines, suggesting that bombesinergic precursor cells in lung may give rise to this disease.*

Approximately 25 percent of all lung cancers are small-cell (oat cell) carcinomas (SCCL), a clinicopathological entity, distinguished from other "non-small-cell" lung cancer histologic types (epidermoid, adenocarcinoma, and large-cell carcinoma) by its characteristic morphology, tendency to metastasize early and widely, frequency of ectopic hormone secretion, and responsiveness to chemotherapy and radiotherapy (*1*). Well-characterized, clonable SCCL tissue culture lines have greatly advanced our knowledge of the biology of SCCL (*2*). These SCCL lines are distinguished from those of the other lung cancer types by the presence of neurosecretory granules, frequent polypeptide hormone secretion, high levels of L-dopa decarboxylase, high levels of the isoenzyme of creatine kinase found in brain, and neuron-specific enolase, as well as a lack of substrate adhesion and characteristic growth factor requirements (*2–4*). Amine precursor uptake and decarboxylating (APUD) cells consist of a widely distributed network of neuroendocrine cells programmed to secrete certain amines and polypeptide hormones (*5*); SCCL and the more benign pulmonary carcinoids are presumed to arise from normal APUD cells in the respiratory tract (*6*). We now report that the 17 SCCL culture lines tested have high quantities of intra-

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cellular bombesin-like immunoreactivity, while the eight "non-small-cell" lung cancer cell (non-SCCL) lines lack detectable amounts of this peptide.

Bombesin is a tetradecapeptide originally isolated from frog skin by Erspamer (*7*) on the basis of its ability to release gastrointestinal hormones. It has become apparent that bombesin-like "neuropeptides" are natural, plentiful components of mammalian brain (*8*), stomach (*9*), intestine (*10*), and fetal lung (*11*). At low (nanogram to microgram) intracerebral doses, this potent biologically active peptide produces hyperglycemia (*12*), anorexia (*13*), brain site–dependent analgesia (*14*), and hypothermia (*14, 15*). In the lung it may have a paracrine effect on bronchiolar muscle, controlling local perfusion and ventilation (*11*). Like other neuropeptides, bombesin (*8*) and its specific cell-surface receptors (*14, 16*) are distributed heterogeneously in brain and gut, the amounts of peptide being undetectable in the cerebellum and highest in the substantia gelatinosa of the spinal cord (5.9 pmole per milligram of soluble protein) (*8*). The distribution pattern of the neuropeptide substance P in the brain is strikingly similar to that of bombesin, although brain is severalfold richer in substance P than bombesin (*17*).

Table 1. Immunoreactive bombesin and substance P concentrations of 25 continuous lung cancer cell lines. The human lung cancer cell lines in general have been described (*2*). All cell lines started by us (NCI lines) and lines from other sources came from patients with the histologic diagnosis of lung cancer of the same cytologic type as the cell line, had human isozymes and chromosomes, grew as tumors in athymic nude mice with the appropriate lung cancer histology, and formed colonies in soft agarose (*2*). The SCCL lines express high specific activity of L-dopa decarboxylase, CK-BB, and neuron-specific enolase, while the non-SCCL lines lack these characteristics. All cell lines were assayed simultaneously for substance P–like and bombesin-like immunoreactivity (*8*). Frozen, washed pellets of approximately 1×10^8 to 5×10^8 cells from each line were extracted with 15 ml of boiling 2*N* acetic acid for 10 minutes and sonicated thoroughly to yield about 10 mg of solubilized protein, which was lyophilized to dryness.

Lung cancer cell line	Peptide concentration* (pmole/mg)	
	BN-like	Substance P–like
SCCL		
NCI-H209	12.7	<0.01†
NCI-N231	6.7	0.16
NCI-H182	5.1	<0.01
NCI-H107	3.8	<0.01
NCI-N408	2.4	<0.01
NCI-H69	1.7	<0.01
NCI-H250	1.35	<0.01
NCI-N390	0.67	0.02
NCI-N464	0.66	0.60
NCI-N220	0.49	0.50
NCI-H64	0.36	<0.01
NCI-H128	0.19	<0.01
NCI-H146	0.17	<0.01
NCI-H123	0.12	<0.01
NCI-H187	0.04	<0.01
OH-1	0.03	<0.01
NCI-H60	0.02	<0.01
Adenocarcinoma		
NCI-H23	<0.01	0.04
NCI-H125	<0.01	<0.01
A549	<0.01	<0.01
Mesothelioma		
NCI-H28	<0.01	0.02
NCI-H226	<0.01	<0.01
Large-cell carcinoma		
NCI-H157	<0.01	<0.01
9812	<0.01	<0.01
Squamous cell carcinoma		
U1752	<0.01	<0.01

*Picomoles per milligram of soluble protein. †< 0.01, not detectable.

We examined extracts of 25 continuous cell lines of lung cancers for the presence of immunoreactive bombesin and substance P (Table 1). Immunoreactive bombesin was readily detectable in all 17 SCCL examined, and the quantities ranged 600-fold from 0.02 to 12.7 pmole per milligram of soluble protein. By contrast, none of the non-SCCL contained detectable levels of bombesin-like immunoreactivity. Much lower quantities of substance P–like immunoreactivity were detected in six different cell lines, four SCCL and two non-SCCL cultures. The levels of bombesin with immunoreactive properties in several SCCL cell lines are high; for example, cell line NCI-H209 has twice the bombesin concentration of the richest area of the brain. By contrast, the levels of substance P detected in the cultures are considerably lower; the concentration of bombesin in NCI-H209 was 1000-fold greater than that of substance P, even though these peptides share sequence homologies at the COOH-terminal, which is where both antiserums are directed. These findings are of interest because they indicate that the technical problems of distinguishing substance P from bombesin have been surmounted, and because they suggest that these two neuropeptides need not be expressed together in the same cell.

Although brain-derived bombesin has yet to be sequenced, its chromatographic properties appear identical with the frog-skin-derived bombesin structure (*9*). Five cell lines with the highest levels of bombesin-like immunoreactivity (NCI-H209, NCI-N231, NCI-H182, NCI-H107, and NCI-N408) were further characterized by chromatography on a Sephadex LH-0 column. All five cell line extracts gave similar results, and Fig. 1 shows the chromatographic profile of one line, NCI-N209 (and an extract of human liver metastases). The significant peak of immunoreactivity from the SCCL lines and the liver co-chromatographed with the tetradecapeptide bombesin standard. Moreover, in experiments with high pressure liquid chromatography on a C_{18}-Microbondpak column and 100 m*M* triethyl ammonium phosphate in 25 percent acetonitrile, SCCL culture line extracts, brain extracts, and the bombesin standard eluted simultaneously (*18*). In extracts of liver

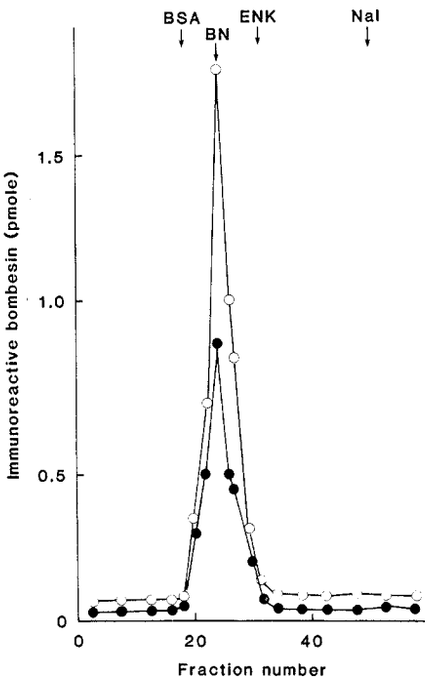

Fig. 1. Immunoreactive bombesin profile after gel filtration of an extract of the SCCL, NCI-209 (○—○), and liver metastases (●—●). Extracts were prepared by boiling tissue for 10 minutes in 2*N* acetic acid and lyophilizing the supernatant after centrifugation (5000*g*). The lyophilized extracts (each approximately 2 mg of soluble protein) were resuspended in 2 ml of a mixture of methanol acetic acid and water (10:2:1) and centrifuged, and the supernatant was applied to a Sephadex LH-20 column (50 by 1.5 cm). The column was eluted with the above methanol mixture, and the fraction size was 1.3 ml. Fractions were diluted in water and then frozen and lyophilized. The lyophilized material was suspended in 50 m*M* tris-HCl, and the *p*H was adjusted to 7.0 before radioimmunoassay. The elution positions of bovine serum albumin [Tyr^4-^{125}I]-bombesin, [^{3}H]leucine-enkephalin, and ^{125}I are indicated. Approximately 90 percent of the applied bombesin immunoreactivity was recovered. Bombesin immunoreactivity measured in SCCL tumor lines, liver specimens from SCCL patients obtained at autopsy, guinea pig brain and bombesin standards did not differ significantly from parallelism. Abbreviations: *BSA*, bovine serum albumin; *BN*, bombesin; *ENK*, enkephalin; *NaI*, sodium iodide.

metastases obtained at autopsy from patients dying of SCCL ($N = 5$), bombesin concentrations were nine times higher than those of extracts of livers from patients dying of other forms of cancer ($N = 4$), (0.18 ± 0.04 compared to 0.02 ± 0.005 pmole per milligram of protein). Thus it is likely that biologically active bombesin is produced by SCCL tumor cells in vivo as well as in vitro. Multiple physiologic effects likely to be produced by bombesin hypersecretion include anorexia, hypothermia, and hyperglycemia.

Fetal lung contains a high concentration of bombesin-like immunoreactivity, and immunocytochemical evidence reveals that bombesin-like immunoreactivity is limited to a subset of endocrine cells of the respiratory epithelium of fetal and neonatal human lung, whose numbers are greatly reduced or even absent in the adult human lung (*11*). The finding of high bombesin-like reactivity in all 17 SCCL cultures examined suggests that SCCL is derived from "bombesinergic" precursor cells, which are plentiful during early development. The fact that we failed to identify a single SCCL cell line lacking bombesin-like immunoreactivity suggests that the presence of bombesin may be an essential property for the continued growth of these cancer cells ["autocrine" factor (*18*)] as well as a clue to the nature of the precursor cell. While many other hormones and neuropeptides can occur in SCCL tumors or cultures (*4*), thus far only bombesin is always present in SCCL and lacking in non-SCCL lines. Finally, it is possible that the presence of bombesin peptides in the blood of people at risk for lung cancer (such as heavy smokers) would allow the early detection of SCCL when treatment with chemo- and radiotherapy is most likely to yield a long-term cure (*19*). Already, we have data indicating that elevated blood levels of bombesin in SCCL patients are correlated with the extent of tumor burden (*20*).

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1 June 1981; revised 27 October 1981

Novel Peptide Neuronal System in Rat Brain and Pituitary

Abstract. *Immunohistofluorescence studies of the rat central nervous system with antibodies to Phe-Met-Arg-Phe-NH_2 (molluskan cardioexcitatory peptide) revealed a widespread neuronal system in the brain, spinal cord, and posterior pituitary. Immunoreactive axons and cell bodies were mainly located in cortical, limbic, and hypothalamic areas. Immunostaining of serial sections of the brain and pituitary showed that the Phe-Met-Arg-Phe-NH_2 immunoreactive neurons were different from neurons labeled by antibodies to either Met-enkephalin or the putative Met-enkephalin precursor Tyr-Gly-Gly-Phe-Met-Arg-Phe, which is structurally related to Phe-Met-Arg-Phe-NH_2. Control staining by antiserum absorption and radioimmunoassay indicated that the antibodies that caused the specific immunofluorescence recognized peptides with an amidated Arg-Phe sequence at the carboxyl terminus.*

The endogenous opiate receptor ligand Met-enkephalin (Tyr-Gly-Gly-Phe-Met, YGGFM) (*1*) is widely distributed throughout the central and peripheral nervous system of vertebrate and invertebrate species (*2*). A number of large Met-enkephalin–containing peptides and proteins, because of their primary structure and anatomical distribution, may serve as precursors for the opioid pentapeptide (*3*). One of these putative Met-enkephalin precursors has the structure Tyr-Gly-Gly-Phe-Met-Arg-Phe (YGGFMRF) (*4*). This peptide is present in rat striata in amounts comparable to those of Leu-enkephalin (*4*, *5*), and, when measured with a specific radioimmunoassay (RIA), its regional distribution in rat brain follows closely that of Met-enkephalin (*6*). The carboxyterminal tetrapeptide fragment of this heptapeptide is strikingly similar to the molluskan cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2 (FMRF-NH_2), which was isolated from clam ganglia by Price and Greenberg (*7*). Because brain contains peptidases capable of cleaving enkephalins at the Gly-Phe bond (*8*), we hypothesized that YGGFMRF might not only serve as a precursor to Met-enkephalin but also to FMRF-NH_2. To test this hypothesis we used antibodies to FMRF-NH_2 for the immunofluorescent staining of rat brain and pituitary sections. These antibodies did indeed detect a widespread neuronal system; however, this neuronal system was unrelated to the Met-enkephalin–YGGFMRF neuronal system.

The antibodies to FMRF-NH_2 were prepared by injecting five rabbits with a carbodiimide reacted peptide-thyroglobulin mixture (*9*) emulsified in Freund's adjuvant. All the rabbits produced serum with a high titer of antibodies. The antiserum from one rabbit (R3-1) was used for immunohistochemical mapping of FMRF-NH_2 immunoreactive neurons in brain, spinal cord, and pituitary. The antiserum from the other four rabbits also specifically labeled the same neurons. The immunofluorescent staining was performed on serial cryostat sections of paraformaldehyde-fixed brains

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