PHILIP MORRIS EUROPE

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To:	Dr. R. Carchman	Date:	January 28, 1998
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Re: Munich studies

Dear Richard,

I was in Munich visiting Richter on Monday and Tuesday this week. The following is a brief update on the current situation:

Publication of results: We shall start writing a manuscript for submission to Carcinogenesis on NNK metabolism in lung tissues. A second manuscript will follow for metabolism of NNK in liver tissues. We both feel that the two data sets should be published separately.

Lung tissue metabolism: We now have data from five different human doners (Figs 1-3). Kinetic analysis of individual tissue samples and means of all data combined clearly shows that NNAL formation predominates and that α-hydroxylation to methylating species (keto acid) is unfavourable at NNK substrate concentrations less than 30 μM. The combined data (Fig. 1) is also presented with 'curve fitting' (Fig. 2). At very low substrate concentrations (0.01-1.0 μM NNK) it is clearly evident that α-hydroxylation to keto acid is not a significant pathway for NNK metabolism (Fig 3). Inspection of data from individual tissue samples indicates a highly variable capacity to convert NNK to NNAL, as evident from the mean±SEM bars. We are going to extend the present data set to 8 different tissues instead of 5 as originally planned. The only published study to investigate human lung tissue metabolism used a single

tissue sample with 238 µM NNK incubated for 24 h – clearly not very informative.

Liver tissue metabolism: We are still having difficulties obtaining 'healthy' human tissues. The last tissue samples were clearly yellow fatty cirrhotic waste, and were not used.

Extension of study: Although not originally planned, I have asked Richter to extend the program to include the SG hamster. My reasons for this are the unpublished negative NNAL bioassay by Hecht and Lijinsky, and a similar negative study published by a Japanese working group. If I have something to put on the table and into the planned Gorrod monograph, maybe I can tease the data out of Hecht.

Labeled NNAL: We are waiting for delivery of the labeled compound from the US in order to complete the study.

Tissue bank: Since we can obtain 20-30 different human lung tissues per week with sufficient donor data (sex, age, diagnosis etc), I have asked Richter to establish a frozen tissue data bank.

In summary, I am still very happy with the progress being made. Knowing what is currently in the published literature, we have made a significant contribution to understanding the science in this very controversial and speculative field of research.

Best regards,

Tony

multi pack day
Smoker
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5 Humanlungen

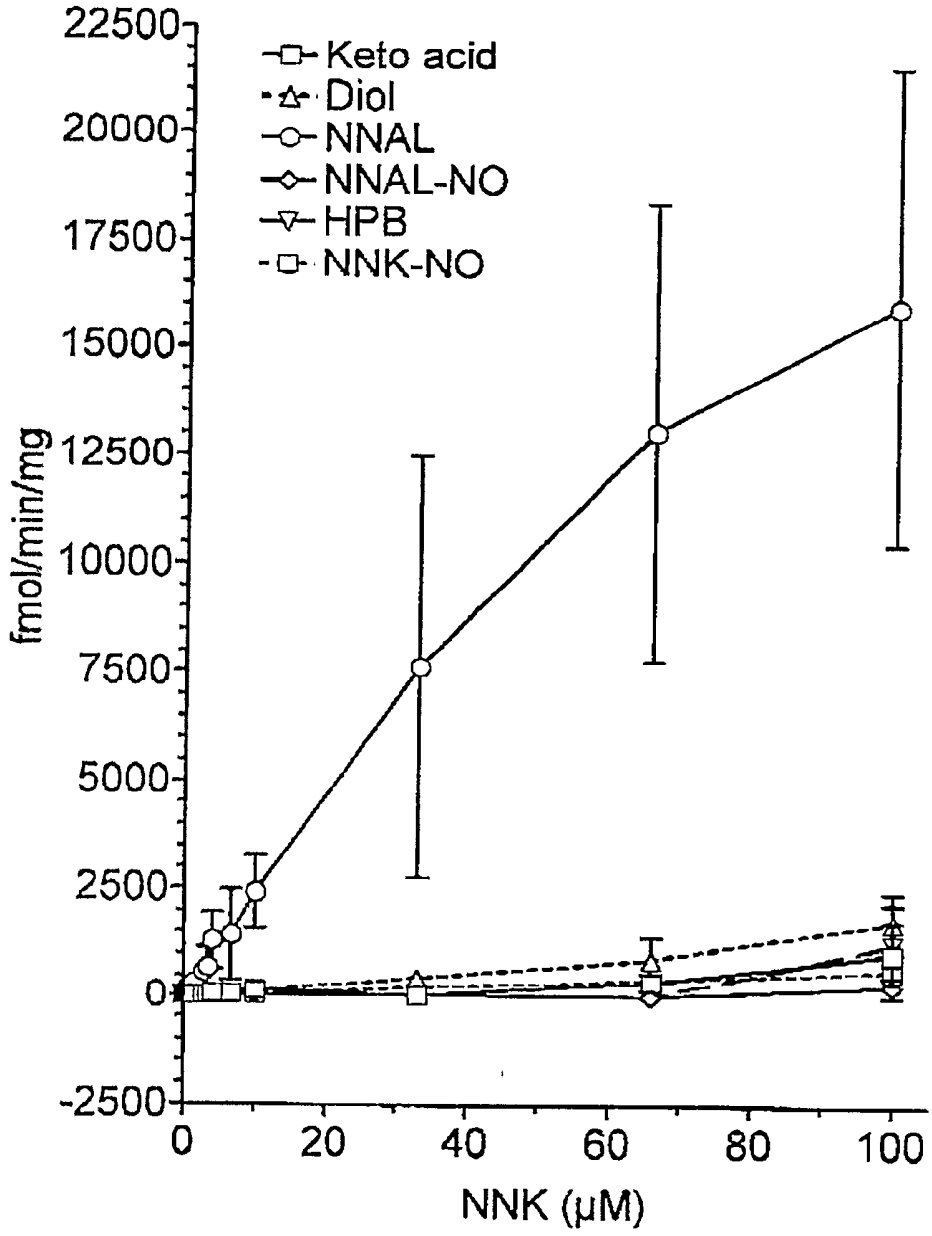

Fig 1. Keto acid derived from α-hydroxylation of NNK
Diol derived from α-hydroxylation of NNAL but not reported to result in adduct formation
HPB derived from α-hydroxylation of NNK and NNN.

5 Humanlungen

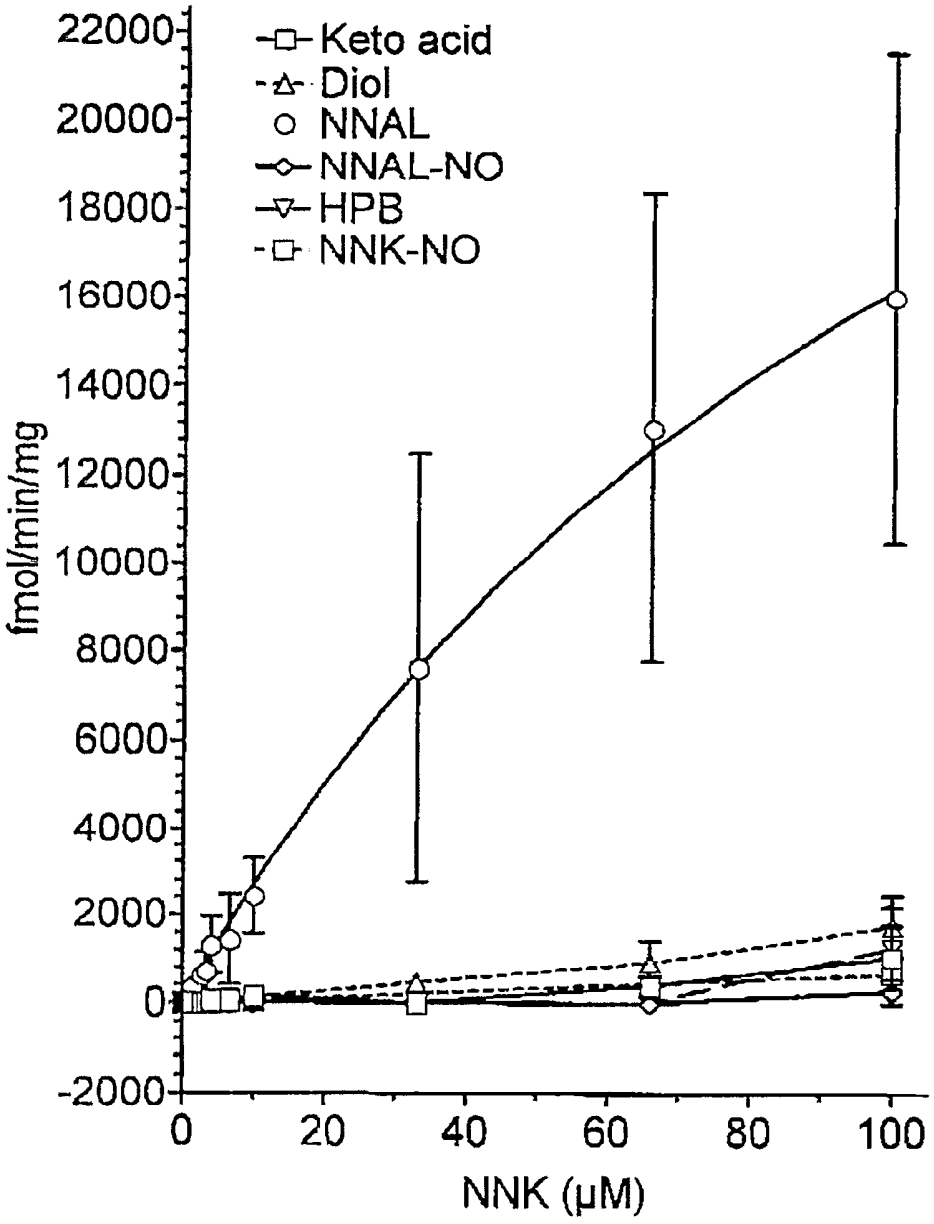

Fig 2.

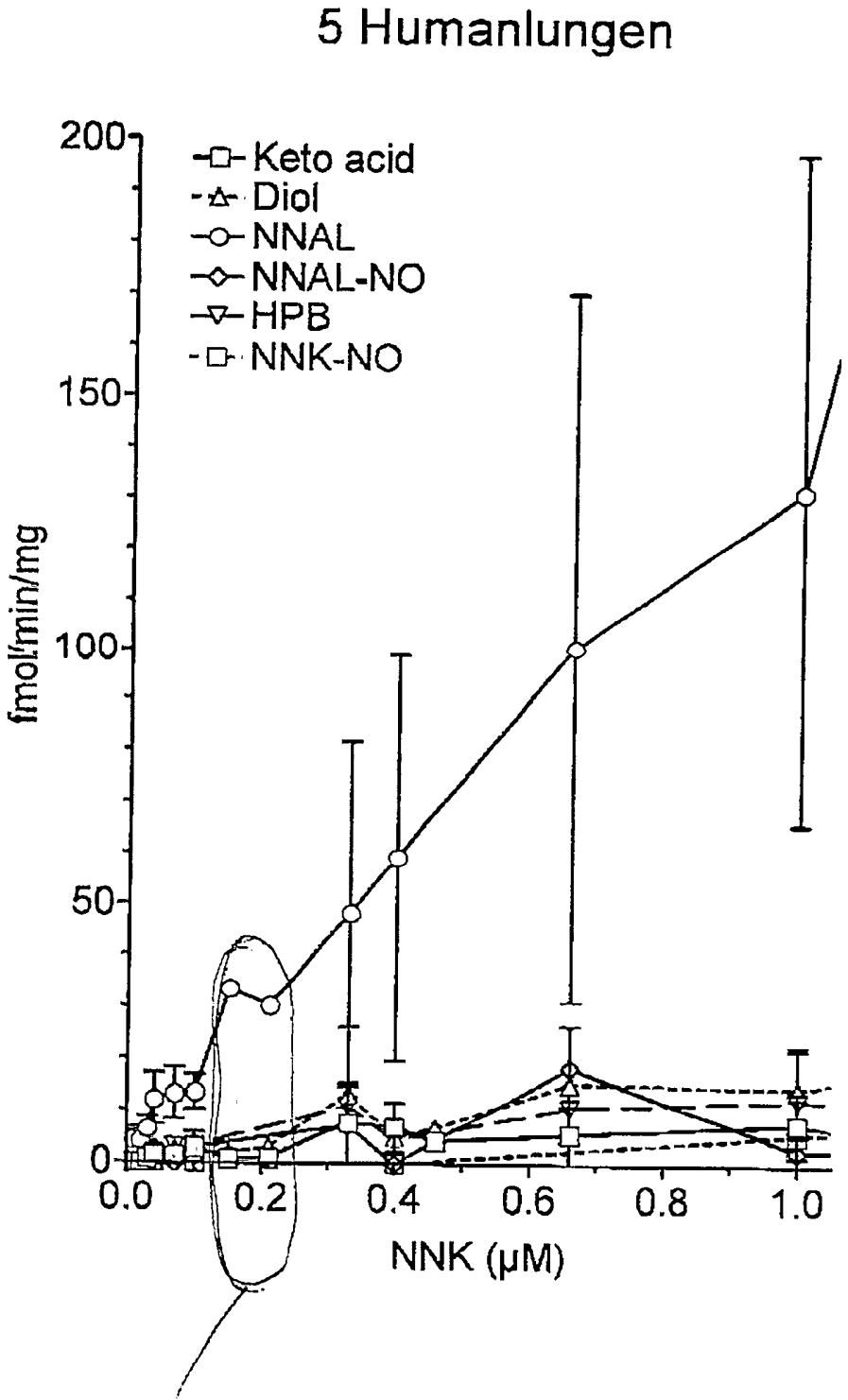

Fig 3.

only determined with one tissue sample.

When we have more data we could blow-up the region from 10 – 200 nM NNK (7 points on curve).

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(SHB)
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Richard: more information from Walter Schlage regarding cytokeratin 20 and its prevalence in lung cancer.
Hans-Jürgen

Table 3. *Distribution of Cytokeratin 20 in Human Tumors as Determined by Immunohistochemistry*[a]

Site	Tumor type	Number of cases tested	CK-20 immunoreactivity[b] –	(+)	+	++	+++	Further CKs usually present[c]
Colon, rectum	Adenocarc., G1, 2	68 (27)[d]	0 (0)[d]	1 (1)	5 (1)	37[e] (17)	25 (8)	8 18 19
	G3, 4	25 (12)	3 (0)	0 (0)	3 (2)	12 (8)	7 (2)	
Stomach	Adenocarc., G1, 2	11 (6)	1 (1)	6 (2)	3 (2)	1 (1)	0 (0)	(7) 8 (17) 18 19
	G3, 4	21 (14)	6 (3)	3 (2)	2 (2)	9 (6)	1 (1)	
Gallbladder, bile ducts	Adenocarc., G1, 2	13 (4)	1 (0)	2 (0)	7 (3)	2 (1)	1 (0)	7 8 (17) 18 19
	G3, 4	6 (2)	2 (1)	0 (0)	2 (0)	2 (1)	0 (0)	
Pancreas	Adenocarc.[g], G1, 2	22 (5)	8 (2)	5[h] (0)	7 (3)	2 (2)	0 (0)	(4) 7 8 (17) 18 19
	G3, 4	23 (11)	11 (5)	3 (2)	8 (4)	1 (0)	0 (0)	
Liver	Hepatocellular carc.	4 (0)	0	4	0	0	0	8 18
Thyroid gland	Follicular (n = 2) and papillary (n = 4) carc.	6 (0)	6	0	0	0	0	7 8 18 (19)
	C-cell carc.	1 (1)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	(7) 8 18 (19)
Salivary glands	Adenoid-cystic carc.	13 (4)	13 (4)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
	Mucoepidermoid tumor	3 (1)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
	Adenocarc.	3 (0)	3 (0)	0 (0)	0 (0)	0 (0)	0 (0)	n.a.
Mammary gland	Invasive ductal carc., G1, 2	92[i] (7)	88 (7)	4 (0)	0 (0)	0 (0)	0 (0)	(7) 8 (14) (17) 18 19
	G3	19 (1)	15 (1)	3 (0)	0 (0)	1 (0)	0 (0)	
	Invasive lobular carc.	32 (2)	30 (2)	2 (0)	0 (0)	0 (0)	0 (0)	7 8 18 19
	Medullary carc.[j]	3 (0)	2	1	0	0	0	n.a.
	Mucinous carc.	4 (0)	3	1	0	0	0	n.a.
Endometrium	Adenocarc.[k], G1, 2	19 (2)	11 (1)	8 (1)	0 (0)	0 (0)	0 (0)	(4) (5) (7) 8 (13) (17)
	G3, 4	3 (1)	3 (1)	0 (0)	0 (0)	0 (0)	0 (0)	18 19
Ovary	Serous, endometrioid, anaplastic and clear-cell tumors[l]	34 (7)	31 (7)	3 (0)	0 (0)	0 (0)	0 (0)	(4) (5) 7 8 18 19
	Mucinous tumors[m]	6 (0)	0	0	1	5	0	(7) 8 19 (19)
Kidney	Renal-cell carc.							
	Clear-cell type	25 (4)	24 (4)	1 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Chromophilic cell type	5 (0)	4	1	0	0	0	(7) 8 18 19
	Chromophobe cell type	9 (0)	8	1	0	0	0	7 8 18 (19)
Prostate gland	Adenocarc.	6 (1)	0 (0)	5 (1)	1 (0)	0 (0)	0 (0)	n.a.
Pleura	Malignant mesothelioma[n]	10 (0)	10	0	0	0	0	(4) 5 7 8 (14) (17) 18 19
Lung	Adenocarc.[o], G1, 2	23 (2)	19 (1)	3 (0)	0 (0)	0 (0)	1 (1)	(4) 7 8 18 19
	G3	10 (2)	9 (2)	1 (0)	0 (0)	0 (0)	0 (0)	
	Squamous cell carc., G1, 2	5 (1)	3 (0)	2 (1)	0 (0)	0 (0)	0 (0)	(4) 5 6 8 (10/11)
	G3	7 (0)	5	0	2	0	0	(13) 14 (15) 16 17 (18) 19
	Small-cell carc.							
	Intermediate-cell type	7 (3)	5 (3)	2 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Oat-cell type	8 (5)	7 (4)	1 (1)	0 (0)	0 (0)	0 (0)	
Skin	Merkel-cell carc.[p]	15 (4)	0 (0)	0 (0)	0 (0)	6 (2)	9 (2)	8 18 (19)
Small intestine, pancreas	Neuroendocrine tumors[q]	7 (3)	4 (2)	3 (1)	0 (0)	0 (0)	0 (0)	8 18 (19)
Urinary bladder, ureter, renal pelvis	Transitional-cell carc., G1, 2	7 (10)	0 (0)	2 (0)	1 (0)	2 (0)	2 (0)	(4) (5) 7 8 13
	G3, 4	17 (11)	1 (1)	2 (2)	0 (0)	10 (5)	4 (3)	(14) (17) 18 19
	Transitional-cell carc. with squamous metaplasia and squamous cell carc.	5 (2)	4 (2)	0 (0)	1 (0)	0 (0)	0 (0)	4 5 6 7 8 (10/11) 13 14 (16) 17 18 19
Cervix uteri	Squamous cell carc.	4 (0)	4	0	0	0	0	(4) 5 6 (8) (10/11) (13) 14–17 (18) 19
Oral cavity, pharynx, larynx	Squamous cell carc.[r]	67 (22)	61 (20)	5 (1)	1 (1)	0 (0)	0 (0)	
Esophagus	Squamous cell carc.	5 (4)	4 (3)	1 (1)	0 (0)	0 (0)	0 (0)	
Skin	Squamous cell carc.	9 (0)	9	0	0	0	0	5 6 (10/11) 14–17

Table 3. (*Continued*)

Site	Tumor type	Number of cases tested	CK-20 immunoreactivity[b]					Further CKs usually present[c]
			–	(+)	+	++	+++	
	Basal-cell carc.	3	3	0	0	0	0	5 (8) 14 (15) 17 (19)
	Malignant melanoma	5 (2)	5 (2)	0 (0)	0 (0)	0 (0)	0 (0)	None [rarely (8) (18)]
Testis, ovary[s]	Germ-cell tumors							
	Seminoma	4 (0)	4	0	0	0	0	None [rarely (8) (18)]
	Embryonal carc.	5 (1)	5 (1)	0 (0)	0 (0)	0 (0)	0 (0)	8 18 (19)
	Yolk-sac tumor	2 (0)	1	1	0	0	0	n.a.
Mesenchymal tumors[t]		10	10	0	0	0	0	None [rarely (8) (18)]

[a] Data are based on findings of immunofluorescence or immunoperoxidase microscopy performed on cryostat sections stained using rabbit antibodies and/or MAbs specific for CK 20.
[b] Scoring was performed according to the proportion of positive tumor cells: –, negative reaction; (+), <5% positive; +, 5%–20% positive; ++, 21%–80% positive; +++, >80% positive.
[c] Data compiled from previous studies based on gel-electrophoresis and/or immunohistochemistry findings (6, 9, 11, 13, 15, 20, 22, 24–30, 37, 46, 52–62). CKs present as minor components or in some but not all cases are indicated in parentheses. Rarely expressed CKs have not been included. n.a., no data available.
[d] First number = total number (primary tumors and metastases); number in parentheses = number of metastases (lymph node and distant).
[e] Including one case of adenocarcinoma of the small intestine.
[f] Including signet-ring-cell carcinomas [3 cases – or (+), 4 cases ++ or +++].
[g] Duct cell type.
[h] Including two cases of adenocarcinoma of the bile papilla.
[i] Including two cases of intraductal carcinoma [1 case –, 1 case (+)].
[j] Partly with portions of invasive ductal carcinoma.
[k] Including one clear-cell carcinoma (G2) and one papillary serous carcinoma (G2; both cases negative). Some adenocarcinomas exhibited squamous metaplasia (see ref. 37).
[l] Including borderline cystadenomas (4 cases, all negative).
[m] Including 1 cystadenoma and 2 borderline cystadenomas.
[n] 5 cases epithelial, 4 cases biphasic, 1 case sarcomatoid.
[o] Including 4 bronchioloalveolar carcinomas [2 cases –, 2 cases (+)] and 2 acenosquamous carcinomas (G3; both negative).
[p] In 8 cases, CK 20 was determined by two-dimensional gel electrophoresis only (++, relatively moderate amounts, 5 cases; +++, relatively large amounts, 3 cases).
[q] Comprising 2 intestinal carcinoids [both (+)] and 5 endocrine tumors of the pancreas.
[r] Various degrees of differentiation and keratinization; the cases scored "(+)" and "+" were predominantly poorly differentiated, nonkeratinizing specimens.
[s] One embryonal carcinomas (with teratoma) and one yolk-sac tumor were from the ovary (both negative).
[t] Including well-differentiated and myxoid chondrosarcoma, leiomyosarcoma, synovial sarcoma (biphasic), high-grade lymphoma, and malignant Schwannoma.

centrated in superficial tumor cells resembling umbrella cells. CK-20 staining could also be demonstrated in paraffin sections (Figure 9b). Notably, tumors of grades 3 and 4 were usually strongly positive (for lymph-node metastases of two such cases, Figure 9c, d). Only 1 of the 24 cases of pure transitional cell carcinoma studied was completely CK-20 negative. Squamous differentiation in tumors resulted in a reduction in the level of CK-20 expression (Table 3).

Of our group of squamous cell carcinomas of the uterine cervix, oropharynx, larynx, esophagus, and skin, which included all grades of malignancy and degrees of keratinization, most cases were negative for CK 20 (Figure 9e), with the rest containing few or few positive cells (Figure 9f, Table 3).

All of the small series of nonepithelial tumors investigated (including primary and metastatic malignant melanomas, various types of sarcomas, and malignant lymphomas) were negative for CK 20 (Table 3).

In selected cases of carcinoma, gel electrophoresis and immunoblotting were performed to obtain direct evidence for the presence of CK 20. At two-dimensional gel electrophoresis, CK 20 could be identified, from its specific coordinates, in adenocarcinomas of the colon,[39] stomach (Figure 10a), and gall bladder (polypeptide "x" in[9]), transitional-cell carcinomas[46] as well as Merkel-cell carcinomas of the skin,[54,63] while no polypeptide corresponding to CK 20 could be recognized in other types of carcinomas and neuroendocrine tumors. The authenticity of the CK 20 resolved by gel electrophoresis was further confirmed by Western blotting. In such experiments using MAbs against CK 20, an immunoreactive protein band of M_r 46,000, definitely representing CK 20, was identified in adenocarcinomas of the colon but not in such tumors of the ovary and breast (Figure 10b, c).

Discussion

General Considerations

Immunologically, the most remarkable result of our study is probably the generation of seven different MAbs spe-

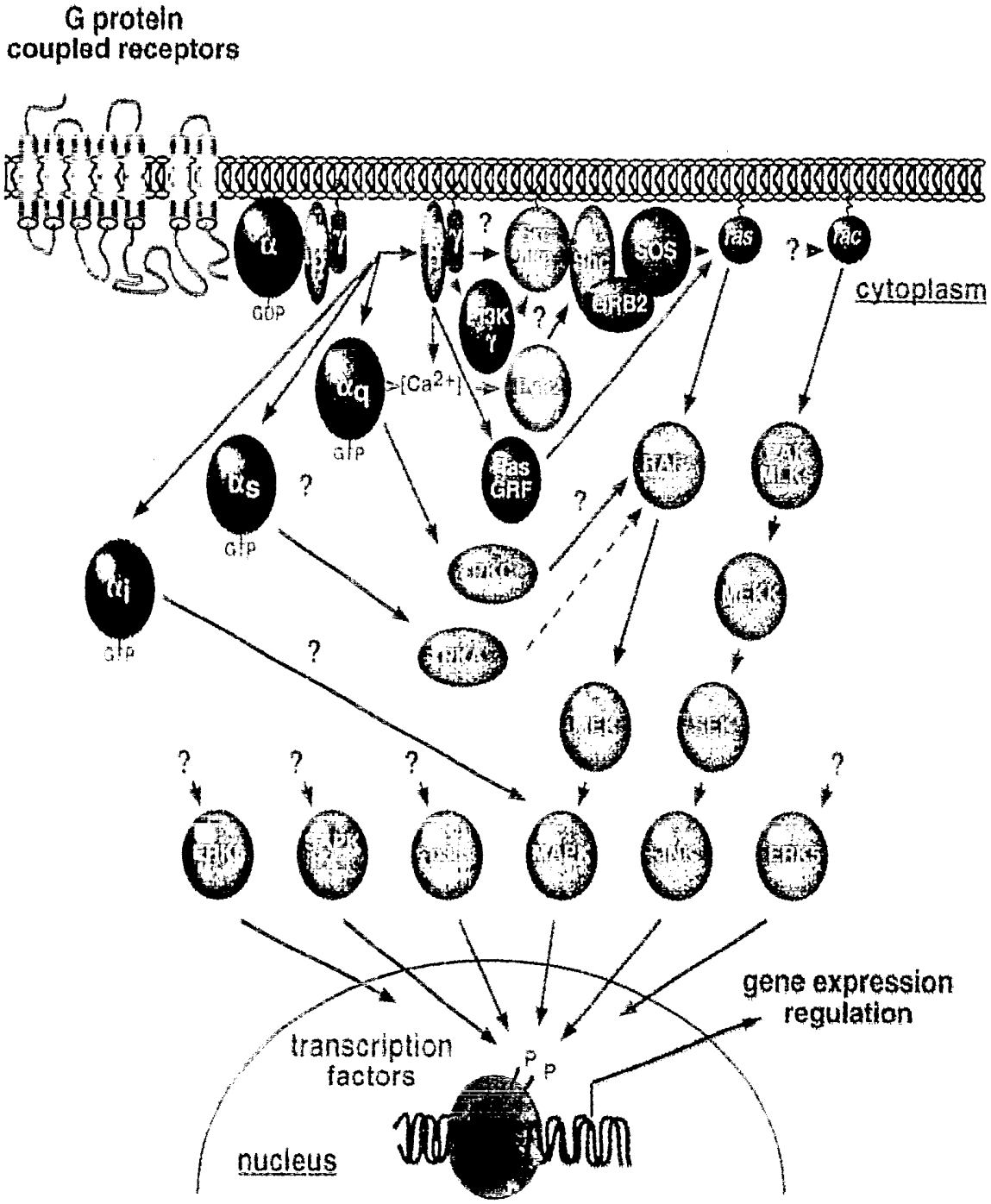

FIG. 2. **Divergent protein kinase cascades link G protein-coupled receptors to the nucleus.** Accumulating evidence suggests that parallel kinase cascades control the activity of members of the MAP kinase family of serine-threonine kinases. (see text for details). The pathway connecting G protein-coupled receptors to low molecular weight GTP-binding proteins and to additional members of the MAP kinase superfamily as well as the identity of biologically relevant targets for these kinase cascades is yet to be fully elucidated.