

## A POLYMERASE-CHAIN-REACTION ASSAY FOR THE SPECIFIC IDENTIFICATION OF TRANSCRIPTS ENCODED BY INDIVIDUAL CARCINOEMBRYONIC ANTIGEN (CEA)-GENE-FAMILY MEMBERS

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**Carcinoembryonic antigen (CEA) is a tumor marker that belongs to a family of closely related molecules with variable expression patterns. We have developed sets of oligonucleotide primers for the specific amplification of transcripts from individual CEA-family members using the reverse transcriptase/polymerase chain reaction (RT/PCR). Specific primer sets were designed for CEA, non-specific cross-reacting antigen (NCA), biliary glycoprotein (BGP), carcinoembryonic antigen gene-family members 1, 6 and 7 (CGM1, CGM6 and CGM7), and one set for all pregnancy-specific glycoprotein (PSG) transcripts. Primers were first tested for their specificity against individual cDNA clones and product-hybridization with internal, transcript-specific oligonucleotides. Total RNA from 12 brain and 63 gynecological tumors were then tested for expression of CEA-related transcripts. None were found in tumors located in the brain, including various mesenchymal and neuro-epithelial tumors. CEA and NCA transcripts were, however, present in an adenocarcinoma located in the nasal sinuses. In ovarian mucinous adenocarcinomas, we always found co-expression of CEA and NCA transcripts, and occasionally BGP mRNA. CEA-related transcripts were also found in some serous, endometrioid and clear-cell ovarian carcinomas. CEA, NCA and BGP transcripts were present in endometrial carcinomas of the uterus and cervical carcinomas, whereas uterine leiomyomas were completely negative. No transcripts were found from CGM1, CGM6, CGM7 or from PSG genes in any of the tumors tested. The PCR data were compared with immunohistochemical investigations of ovarian tumors at the protein level using CEA (26/3/13)-, NCA-50/90 (9A6FR) and NCA-95 (80H3)-specific monoclonal antibodies.**

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Tumor markers are usually defined as molecules produced by malignancies, whose presence or altered serum concentration, as compared with normal values, gives an indication that a tumor exists. Carcinoembryonic antigen (CEA) is a widely used human tumor marker for a variety of carcinomas (reviewed in Shively and Beatty, 1985). It is a membrane-bound glycoprotein that is also released into the blood and other body fluids. For this reason, not only are post-operative CEA serum levels a useful parameter for monitoring recurrent tumors, but the presence of CEA on the surface of tumor cells also offers the possibility of localizing tumors with CEA-specific, radiolabelled antibodies and targeting in immunotherapy. CEA is not a tumor-specific antigen, but it has only a low steady-state concentration in normal tissues.

CEA is a member of a family of glycoproteins with variable expression patterns. CEA-related antigens are present in different normal tissues: for example, non-specific cross-reacting antigen (NCA) is found in granulocytes, while biliary glycoprotein (BGP) is present in bile (reviewed in Thompson *et al.*, 1991). The protein backbone, as determined by protein microsequencing or as derived through molecular cloning, reveals high sequence similarity among individual members of the CEA family. A gene family exists, comprising at least 22 genes (Khan *et al.*, 1992) closely clustered on the long arm of chromosome 19 (Thompson *et al.*, 1991). Sequence comparisons allow a division into 2 main sub-groups, one encoding the classical CEA and cross-reacting antigens, the other the

pregnancy-specific glycoproteins (PSGs) expressed in increasing amounts by the placenta during pregnancy.

A major problem in studying the expression of CEA-related antigens at the protein level has been the lack of well-characterized antibodies that are specific for individual antigen species. Through molecular cloning and the creation of stable transfectants expressing individual members of the CEA family, panels of monoclonal antibodies (MAbs) have been tested using FACscan analyses to better define their specificities (Daniel *et al.*, 1993). The mass of sequence data produced in the last few years has also made it possible to study the expression patterns of individual members at mRNA level. Transcripts have been found by cDNA cloning studies and DNA/RNA hybridization (Northern) analyses for 17 CEA-related genes (reviewed in Thompson *et al.*, 1991; Khan *et al.*, 1992), yet the assignment of each CEA-related antigen to a specific gene remains difficult. Nevertheless, transcripts have been assigned to CEA (Oikawa *et al.*, 1987), NCA-50/90 (Neumaier *et al.*, 1988), NCA-95 (Berling *et al.*, 1990) and BGP (Hinoda *et al.*, 1988).

Although the CEA cross-reacting antigens are expressed in various normal tissues, they may also be useful as tumor markers. For example, PSGs have been used as markers for choriocarcinomas (Sakuragi, 1982). A 3.9-kb transcript for BGP has been reported not only in normal human hepatocytes but also in 16/21 hepatocellular carcinomas (Hinoda *et al.*, 1990). NCA but not CEA transcripts are more prevalent in benign adenomas and colonic carcinomas than in adjacent normal mucosa, indicating the potential use of NCA as a marker for progression towards malignancy of colonic tumors (Chi *et al.*, 1991). In contrast, CEA and BGP transcripts have recently been reported to be more prevalent in certain lung tumors than in corresponding normal tissues (Kim *et al.*, 1992). We here examine in more detail the potential usefulness of individual members of the CEA family as tumor markers and attempt to determine their expression patterns more exactly.

It is usually necessary to use labelled DNA fragments to visualize transcripts using the DNA/RNA hybridization method. However, due to their high sequence similarities, it is not possible to differentiate all CEA-related transcripts with DNA fragment probes. On the basis of existing sequence data, we describe here the design and testing of oligonucleotide primer pairs to identify specific CEA-related transcripts in human tumors, using the reverse transcriptase/polymerase chain reaction (RT/PCR). The presence or absence of given transcripts has been correlated with the presence or absence of corresponding antigens as indicated by MAbs specific for CEA, NCA-50/90 and NCA-95.

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## MATERIAL AND METHODS

## Oligonucleotide primers

Sequence comparisons of individual CEA-gene-family members were carried out using published sequences to design oligonucleotides specific for CEA, NCA, BGP, CGM6, CGM1, CGM7 and for PSG1-PSG15 (Table I). A set of oligonucleotide primers was also synthesized for amplification of all known CEA-gene-family members. In order to achieve this, the program "Primer" was applied (Lucas *et al.*, 1991). This program was developed in our group for the comparison and identification of specific nucleotide sequences from gene families.

## Source of cDNA clones

A full-length CEA cDNA clone was constructed (Pèlerin *et al.*, 1992), sub-cloned into a Bluescript M13<sup>+</sup>KS vector (Stratagene, La Jolla, CA) and linearized prior to PCR analysis by HindIII digestion. A 2-kb EcoRI fragment of the cDNA clone pNCA1 (Neumaier *et al.*, 1988) containing the coding region of NCA and a BamHI/EcoRI fragment from the partial BGP cDNA clone 18 (Hinoda *et al.*, 1988), both kindly made available by Dr. J. Shively (Beckman Research Institute, Duarte, CA) were also used for primer specificity testing. For CGM6, a full-length cDNA clone in the Bluescript vector (Berling *et al.*, 1990) was linearized using EcoRI, as was the PSG3 cDNA clone pSP-1i (Rooney *et al.*, 1988), which was a kind gift from Dr. N. Hardman (Ciba-Geigy, Basel, Switzerland). A CGM7 cDNA clone which was provided by Dr. M. Kuroki (Fukuoka, Japan) and a cDNA clone containing the complete coding region of CGM1a (Nagel *et al.*, 1993) were also used for primer testing.

## Tumor source, RNA extraction and polymerase-chain-reaction conditions

The tumors located in the brain and relevant clinical data were kindly provided by Dr. Seeger (Department of Neurosurgery, University Hospital, Freiburg, Germany). The gynecological tumors and clinical data were from Dr. T. Bauknecht (Department of Gynecology, University Hospital, Freiburg, Germany) and Dr. J. Arsenau (Department of Pathology,

Royal Victoria Hospital, Montreal, Canada). Tumor material was quick-frozen in liquid nitrogen and stored at -70°C prior to RNA isolation from 0.5 to 1 g tumor tissue, using the acid phenol extraction method described by Chomczynski and Sacchi (1987) or the guanidinium-isothiocyanate extraction procedure (Sambrook *et al.*, 1989). Tissues were first ground under liquid nitrogen with a pestle and mortar and finely homogenized in extraction buffer using a tight-fitting glass/Teflon homogenizer.

Reverse transcription and polymerase chain reactions were slightly modified after Sambrook *et al.* (1989), as described in Lucas *et al.* (1991). For the reverse transcription step, either 25 pmole of the selected 3' oligonucleotide or later 50 pmole of a random hexamer mixture (Pharmacia, Uppsala, Sweden) were used to prime first-strand DNA synthesis from 200 ng or 1 µg of total RNA. The optimized system utilized 50 pmole of random hexamer and 1 µg of total RNA for reverse transcription. The specific oligonucleotides (50 pmole each) were added after the reverse-transcription step, and 30 amplification cycles were applied using optimal annealing temperatures (Table I) for the given primer sets (15 sec/step).

Prior to testing for the presence of CEA-related transcripts, the integrity of the RNA extracted from various tumors was determined. This control was to rule out false negative results where the RNA may be degraded, or reverse transcription and/or DNA amplification inhibited due to the presence of impurities. It was achieved by PCR using a primer set to amplify β-actin transcripts. This was tested both with and without reverse transcriptase, so as to avoid false positive signals due to contaminating DNA in the preparations. It is known that a large number of processed β-actin pseudogenes exists (Ng *et al.*, 1985), and, in the absence of the reverse transcription step, positive signals of the same size as from the mRNA reveal DNA contamination. However, even in the presence of contamination, differences in signal strength or banding pattern can still be used to confirm RNA integrity. As no intronless pseudogenes have so far been discovered for the CEA-gene family, DNA contamination should not create problems with the PCR. In the search for another ubiquitously

TABLE I - PRIMER SETS FOR SPECIFIC AMPLIFICATION OF CEA-RELATED AND CONTROL TRANSCRIPTS BY RT/PCR

Transcripts	Primer pairs	Sequences	Exon	Position from ATG [nc]	Fragment length [bp]	T <sub>annealing</sub> [°C]	Reference
β-actin	PCRβactin5' PCRβactin3'	5'-ACGGCTGCTTCCAGCTCCTC-3' 5'-AGCCATGCCAATTCATCTTGT-3'	3	685 ⇒ 706	525	64	Ponte <i>et al.</i> , 1984; Ng <i>et al.</i> , 1985
c-abl	PCRABL1B5' PCRABL3'	5'-ATGGGGCAGCAGCCTGGAAA-3' 5'-GTGATGTAGTTGCTTGGGAC-3'	1b internal	1 ⇒ 20 388 ⇒ 407	407	60	Bernards <i>et al.</i> , 1987; Fainstein <i>et al.</i> , 1989
All CEA-family transcripts	PCRCEAall5' PCRCEAall3'	5'-GGCAGAGGCTCCTGCTCAC-3' 5'-TCAGCAGGGATGCATTGG-3'	5'/L L/N	44 ⇒ 62 308 ⇒ 325	282	56	listed in Thompson <i>et al.</i> , 1991
CEA	PCRCEA-5' PCRCEA-3'	5'-CCATGGAGTCTCCCTCG-3' 5'-GTAGCTTGCTGTGTCATTTC-3'	5'/L A1	-2 ⇒ 15 620 ⇒ 639	641	56	Oikawa <i>et al.</i> , 1987
NCA	PCRNCA-5' PCRNCA-3'	5'-TTCTTCTACTCGCCACAAC-3' 5'-GTTCTTTTGACGCTGAGTA-3'	L/N A	152 ⇒ 171 606 ⇒ 625	474	58	Neumaier <i>et al.</i> , 1988
BGP	PCRBBP-5' PCRBBP-3'	5'-AGGTGAAGACAGGGCCAGC-3' 5'-ATGTTCCATTGATAAGCCAG-3'	5'.L B1	-29 ⇒ -11 811 ⇒ 830	859	56	Hinoda <i>et al.</i> , 1988
CGM6	PCRCGM6-5' PCRCGM6-3'	5'-ATCTCAGCCCCCTCCTGCAG-3' 5'-CAGTGTAGCCACGAGGGTC-3'	5'/L N	10 ⇒ 29 181 ⇒ 200	191	64	Berling <i>et al.</i> , 1990
CGM1	PCRCGM1-5' PCRCGM1-3'	5'-GGAATTCCTGGAGCCAGGCTCTTT-3' 5'-CTGTCGAGGTCTCCACA-3'	5'/L 3'-UTR	-56 ⇒ -35 920 ⇒ 936	992	54	Kuroki <i>et al.</i> , 1991
CGM7	PCRCEAall5' PCRCEAall3'	5'-GGCAGAGGCTCCTGCTCAC-3' 5'-CAGTTGCTTGGTCAGAGTCG-3'	5'/L N	44 ⇒ 62 387 ⇒ 406	363	58	Kuroki <i>et al.</i> , 1991
All PSG transcripts	PCRPSGall5' PCRPSGall3'	5'-AGTTTCTGGATCCTAGGCT-3' 5'-GATGTAATGTAATGGTAGAGG-3'	5'/L N	-56 ⇒ -38 222 ⇒ 242	298	56	listed in Thompson <i>et al.</i> , 1991

The positions of the CEAall and PSGall primer sets are based on the sequences for CEA (Oikawa *et al.*, 1987) and PSG3 (Rooney *et al.*, 1988), respectively. The primer PCRCGM1-5' has an EcoRI linker at the 5'-end (bold print indicates extended sequence not found in CGM1). For exon nomenclature of CEA-gene family, see Thompson *et al.* (1991). nc, nucleotides; bp, base pairs.

expressed control mRNA, where no processed pseudogenes exist, we synthesized a primer set (Table I) for the amplification of the 1b transcripts for the proto-oncogene *c-abl* (Bernards *et al.*, 1987).

For each primer set, positive and negative controls were included in the RT/PCR reaction. As a negative control, water instead of RNA was used in all cases. For the CEAall, CEA and NCA sets, we initially used RNA from a human colonic tumor and later from an ovarian mucinous tumor as positive controls. Total RNA from leukocytes of a chronic myeloid leukemia patient and later RNA from transfectants expressing the respective CEA-family members were the positive controls for the BGP, CGM1, CGM6 and CGM7 primer sets. Alternatively, for the BGP set, RNA from the human adenocarcinoma cell line HT29 was also used. The positive control for the PSGall primer set was total RNA from a term placenta.

#### Transfer of DNA and oligonucleotide hybridization

After size separation of DNA fragments on agarose gels, alkaline transfer to Biodyne B membranes (PALL, Dreieich, Germany) was carried out according to Chomczynski and Qasba (1984). The following oligonucleotides were synthesized as specific internal probes to unequivocally identify each amplification product:

CEA/PSGall	5'-CCTCACTTYTMAMCTTCTGG-3'	(max. 1 mismatch)
CEA-specific	5'-GGGATGCACCATCTGTGGG-3'	(≥ 3 mismatches)
NCA-specific	5'-GCTGTAACCAATACGAT-3'	(≥ 3 mismatches)
BGP-specific	5'-CATGGATTTCAGTAGTGAGCTGG-3'	(≥ 2 mismatches)
CGM6-specific	5'-GATGCGCCATCTGCAGGAAG-3'	(≥ 2 mismatches)
CGM1-specific	5'-TGCGACACTGAGCGGCATGGAT-3'	(≥ 3 mismatches)
CGM7-specific	5'-CCCTTGTGCCAATAATA-3'	(≥ 5 mismatches)
PSGall-specific	5'-TAATGTAATGGTAGAGGTC-3'	(≥ 5 mismatches)

The sequence source for each oligonucleotide is as listed in Table I. The minimal number of mismatches to other members of the CEA gene family are indicated in brackets. The CEA/PSGall oligonucleotide was designed to hybridize with all family members with maximally 1 mismatch. For the PSGall-specific probe, maximally 2 mismatches were found to all PSG sub-group members. Oligonucleotides were 5'-end-labelled, hybridized (Sambrook *et al.*, 1989) and washed to approximately 4°C below their theoretical melting temperatures, as calculated according to Lathe (1985), in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na-phosphate, pH 7.4, and 1 mM EDTA), 0.1% SDS. The maximal wash temperatures for the CEA/PSGall- and PSGall-specific oligonucleotides took into account a maximum of 1 or 2 mismatches respectively.

#### Immunohistochemistry

Frozen tumor material was embedded in Tissue Tek Freeze medium (Reichert-Jung, Vienna, Austria) and sectioned on a cryomicrotome (Model 2700 Frigocut, Reichert-Jung). Sections of 7 to 10 µm were stored on poly (L-lysine) coated slides at -70°C, and parallel sections were stained with hemalaun/eosin for identification of tissue structure. Prior to freezing, the sections were dried under vacuum at room temperature or 30°C for 1 to 2 hr and fixed for 10 min in acetone. Thawed sections were air-dried and re-hydrated in PBS. Endogenous peroxidase activity was blocked by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Pre-incubation with 3% ovalbumin, Grade II (Sigma, St. Louis, MO) was carried out before the antibody incubations were made. Monoclonal antibodies (Daniel *et al.*, 1993) were used to differentiate CEA (26/3/13), NCA-50/90 (9A6FR) and NCA-95 (80H3) using the avidin-biotin (ABC) procedure and a Vectastain kit (Vector, Burlingame, CA). The first antibodies were diluted to a concentration of 5 µg/ml PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) for incubation. The rabbit anti-mouse IgG bridge antibodies were used at a dilution of 15

µg/ml PBS and the avidin-biotin complex was diluted 1:125 in PBS. Counterstaining was carried out with hemalaun. Positive controls for CEA and NCA-50/90 were colonic tumor, or normal colon sections. For NCA-95, the positive control was a human blood smear. As negative control, the first antibody was either omitted from the buffer or replaced by mouse IgG. The specificity of the antibodies has been tested elsewhere in FACscan analyses, using stable transfectants expressing CEA, NCA-50/90, CGM6 (NCA-95), BGP, CGM1 and CGM7 (Daniel *et al.*, 1993).

## RESULTS

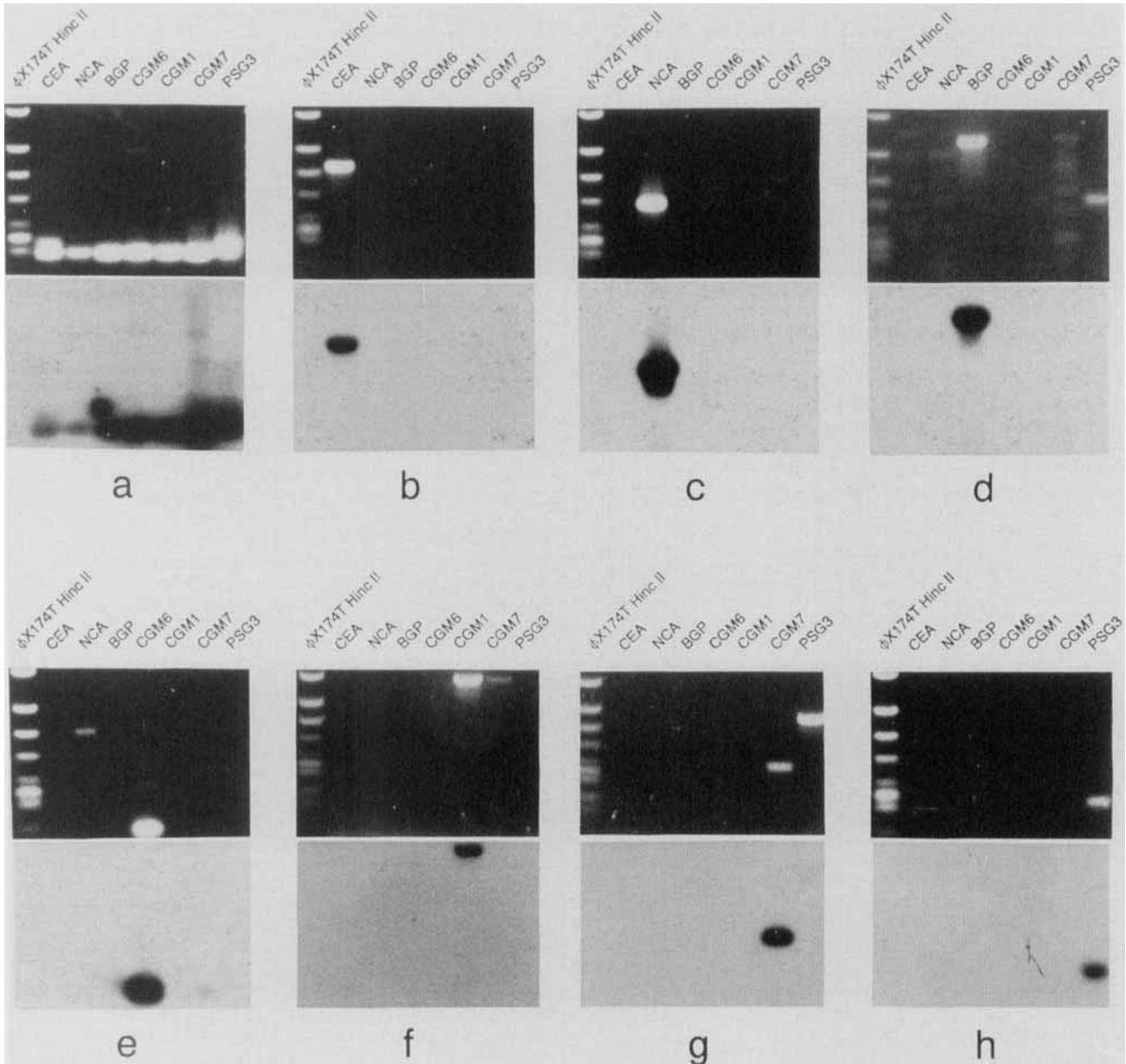
### Development of a PCR system for identification of transcripts derived from CEA-gene-family members

In order to synthesize specific or common oligonucleotide primer sets it was necessary to compare all known CEA-gene-family DNA sequences. To be certain that the amplification products originated from tumor RNA and not from contaminating genomic DNA, the 5' and 3' oligonucleotides were chosen from different exons (Table I).

The different primer sets were then tested and their annealing temperatures optimized for their specificities against various CEA-related cDNAs (Fig. 1). One primer set (PCRCEAall5'/PCRCEAall3') was designed to amplify mRNA of all CEA-gene-family members and an amplification product of the expected size was found for all cDNA clones tested (Fig. 1a). In all cases, amplification fragments of the expected sizes were seen (Table I). Despite theoretical specificity of the primer sets designed to amplify only individual members of the CEA-gene family, when tested against all cDNA clones, some cross-amplification with products of the same or different sizes was found Fig. 1, c-g. However, higher specificity was achieved by transferring the amplification products to nylon membranes and by hybridization with internal oligonucleotides specific for each product with their respective cDNA templates.

The *c-abl* primer set as a control for the integrity of the RNA was tested against total genomic DNA where no amplification signals were seen (data not shown) and optimized for amplification of total RNA extracts. In order to get visible signals for the *c-abl* transcript, 1 µg of total RNA per PCR was needed. For determination of the optimal amounts of total RNA needed to give visible signals in the PCR reaction for CEA-related transcripts, variable amounts of RNA from a human colon adenocarcinoma were tested. Although signals were still visible with 1 to 10 ng RNA for CEA and NCA, we used 200 ng total RNA for the first screenings. However, after comparing the results gained with the immunohistochemical staining patterns from the same tumors, it became obvious that the latter showed greater sensitivity. For this reason, and for uniformity with the *c-abl* control, we then increased the amount of total RNA to 1 µg/reaction.

Once the PCR conditions had been optimized, total RNA from selected tumors expected to be either positive (various carcinomas) or negative (different brain tumors), was tested for the presence of CEA-related transcripts (Table II). In order to avoid screening each RNA with all primer sets, after determining the integrity of the RNA, a primer set was used which should amplify all CEA-related transcripts (Fig. 1a). Those RNAs that were negative with this primer set were not further tested with specific oligonucleotides. Three carcinomas revealed expression products of the expected size for CEA and NCA: a squamous carcinoma of the lung, an adenocarcinoma of the pancreas (these were included as positive controls) and an adenocarcinoma located in the nasal sinuses. No transcripts were found for BGP, CGM6 or the PSG sub-group. CGM1 and CGM7 transcripts were not determined. As expected, none of the neuroepithelial (n = 4) or the mesenchymal brain tumors



**FIGURE 1** – Specificity control of primer sets designed to amplify transcripts from individual CEA-gene-family members. PCR amplification was carried out using cDNA clones as templates from individual members of the CEA-gene family (listed above). A HincII digest of bacteriophage  $\phi$ X174T was used as a size marker. The following oligonucleotide primer sets (listed in Table I) were used: *a*, PCRCEAa1; *b*, PCRCEA; *c*, PCRNCA; *d*, PCRBGP; *e*, PCRCEA; *f*, PCRCEA; *g*, PCRCEA; *h*, PCRCEA. Ethidium-bromide staining of the DNA after separation on a 1.8% agarose gel is shown in the upper section and the hybridization results in the lower section of each sub-figure, using the following 5'-end-labeled, internal oligonucleotides: *a*, CEA/PSGall; *b*, CEA-specific; *c*, NCA-specific; *d*, BGP-specific; *e*, CGM6-specific; *f*, CGM1-specific; *g*, CGM7-specific; *h*, PSGall-specific.

( $n = 6$ ) tested contained transcripts for any CEA-gene-family member (Table II).

#### Identification of CEA-related transcripts in gynecological tumors

A larger-scale RT/PCR-screening was carried out on 63 different gynecological tumors (Fig. 2; Table III). The integrity of the RNA was first tested using the  $\beta$ -actin primer set, with and without the reverse transcription step. In some cases, DNA contamination revealed amplification products with and

without reverse transcription (Fig. 2*d*: colon-tumor metastasis). However, the larger fragment representing the active  $\beta$ -actin gene with intervening intron sequences was visible only in the absence of reverse transcription. As this fragment usually disappeared after inclusion of reverse transcription, intact RNA must also be present in such RNA samples. Parallel controls with the *c-abl* primer set confirmed these results (data not shown). In cases where the RNA was intact, the expression of CEA and CEA-related transcripts was

TABLE II - DETECTION OF CEA-GENE-FAMILY TRANSCRIPTS IN SELECTED TUMORS USING AN RT/PCR ASSAY

1 µg total RNA	c-abl/β-actin	CEAall	CEA	NCA	BGP	CGM6	CGM1	CGM7	PSGall
Epithelial tumors									
Breast carcinomas	2/2	0/2	—	—	—	—	—	—	—
Pancreas adenocarcinoma	1/1	1/1	1/1	1/1	0/1	0/1	nd	nd	0/1
Lung squamous carcinoma	1/1	1/1	1/1	1/1	0/1	0/1	nd	dn	0/1
Neuro-epithelial tumors									
Astrocytomas	2/2	0/2	—	—	—	—	—	—	—
Oligodendroglioma	1/1	0/1	—	—	—	—	—	—	—
Glioblastoma multiforma	1/1	0/1	—	—	—	—	—	—	—
Intracranial epithelial tumor									
Nasal sinus adenocarcinoma	1/1	1/1	1/1	1/1	0/1	0/1	nd	nd	0/1
Mesenchymal brain tumors									
Meningeomas	4/4	0/4	—	—	—	—	—	—	—
Hemangioblastoma	1/1	0/1	—	—	—	—	—	—	—
Non-Hodgkin lymphoma	1/1	0/1	—	—	—	—	—	—	—
Metastases in brain									
Lung squamous carcinoma	1/1	0/1	—	—	—	—	—	—	—
Ovarian carcinoma	1/1	0/1	—	—	—	—	—	—	—

nd, not determined

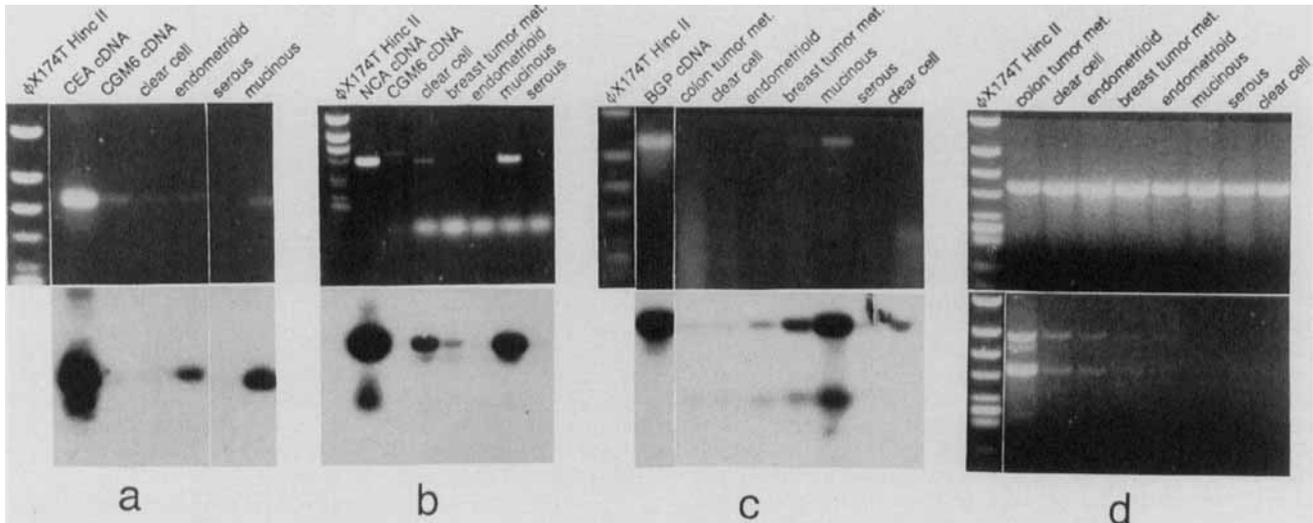


FIGURE 2 - Identification of CEA-related transcripts in ovarian tumors. RNA isolated from different tumors was reverse transcribed and PCR-amplified using the following oligonucleotide primer sets: a, PCRCEA; b, PCRNCA; c, PCR BGP; d, PCRβactin, to specifically amplify CEA, NCA, BGP and β-actin transcripts respectively. Examples are shown from different tumor types (listed above) along with positive controls from respective cDNA clones. The upper part of the figure shows ethidium-bromide staining of DNA gels; the lower part shows hybridization results with the following internal oligonucleotide probes: a, CEA-specific; b, NCA-specific; c, BGP-specific. In d, the upper part of the figure is with reverse transcription and the lower part is without this step.

investigated. As seen in Table III, co-expression of CEA, NCA and occasionally BGP transcripts, but of no other CEA-gene-family member, was found in the mucinous ovarian carcinomas (n = 10), whereas CEA-related transcripts were found only in 4/12 serous, 4/7 endometrioid and 2/7 clear-cell ovarian carcinomas. From 200 ng total RNA, benign leiomyomas of the uterus revealed no CEA-related transcripts (n = 7), whereas some endometrial adenocarcinomas as well as carcinomas of the cervix expressed transcripts for CEA (2/6), NCA (2/6) and BGP (2/6). For unequivocal identification of the different amplification products, the fragments were transferred to nylon membrane and hybridized at high stringency with transcript-specific oligonucleotides (Fig. 2a-c). Although the lower hybridization signal seen in Figure 2c corresponds in size with the cross-amplification product seen with this BGP primer set in the ethidium-bromide-stained gel using the PSG3 cDNA as a control (Fig. 1d), it does not represent a PSG, since the same tumours as shown in Figure 2c were negative when

tested with the PSGall primer set (data not shown). None of the tumors examined revealed the presence of transcripts for CGM1, CGM6, CGM7 or any PSG-sub-family member in the conditions used (data not shown).

*Expression of CEA, NCA-50/90 and NCA-95 in gynecological tumors using specific MAb*

Apart from the internal positive and negative controls, the efficacy of the PCR system was tested at the antigen level using specific MAb in standard immunohistochemical studies to differentiate CEA (Mab 26/3/13), NCA-50/90 (Mab 9A6FR) and NCA-95 (Mab 80H3). A typical staining pattern of these 3 Mabs is shown on serial sections of an ovarian mucinous carcinoma in Figure 3. The CEA- (Fig. 3a) and NCA50-90- (Fig. 3b) specific antibodies both reveal positive staining of tumor cells, whereas NCA-95 is present only in granulocytes (Fig. 3c) and not in tumor cells in this example. The negative control reveals no unspecific background staining (Fig. 3d).

TABLE III - DETECTION OF CEA-GENE-FAMILY TRANSCRIPTS IN GYNECOLOGICAL TUMORS USING AN RT/PCR ASSAY

	c-abl/ β-actin	CEAall	CEA	NCA	BGP	CGM6	CGM1	CGM7	PSGall
Ovarian tumors (1 μg total RNA)									
Benign tumors									
Cystoma	1/1	0/1	—	—	—	—	—	—	—
Malignant tumors									
Carcinomas									
Serous	12/16	4/12	1/4	1/4	1/4	0/4	0/4	0/4	0/4
Mucinous	10/10	10/10	10/10	10/10	4/10	0/10	0/10	0/10	0/10
Endometrioid	7/10	4/7	1/4	1/4	1/4	0/4	0/4	0/4	0/4
Clear-cell	7/8	2/7	2/2	2/2	1/2	0/2	0/2	0/2	0/2
Sarcoma	1/1	0/1	—	—	—	—	—	—	—
Teratomas	2/2	0/2	—	—	—	—	—	—	—
Metastases in ovary									
Colon carcinomas	3/3	2/3	1/2	1/2	1/2	0/2	0/2	0/2	0/2
Stomach carcinoma	1/1	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1
Breast carcinoma	1/1	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1
Uterine tumors (200 ng total RNA)									
Benign tumors									
Leiomyomas	7/7	0/7	—	—	—	—	—	—	—
Malignant tumors									
Endometrial adenocarcinomas	6/6	4/6	2/4	2/4	2/4	0/4	0/4	0/4	0/4
Cervical tumors (200 ng total RNA)									
Carcinomas	3/3	2/3	2/2	2/2	1/2	0/2	0/2	0/2	0/2
Other tumors (200 ng total RNA)									
Vulvar intra-epithelial neoplasia	1/1	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1
Fallopian-tube carcinoma	1/1	0/1	0/1	—	—	—	—	—	—

The total results of the immunohistochemical analyses are compared with those from PCR studies for individual tumors in Table IV. The results for CEA expression at the mRNA and the antigen level correlate well in 17 from 19 ovarian tumors analyzed. For NCA-50/90, the tumors positive at the mRNA level were also positive at the antigen level. However, NCA-50/90 and NCA-95 antigen were found in 5/19 and 4/19 tumors, respectively, that were apparently negative for the corresponding transcripts. In all those cases, these NCA species were located focally in only a few tumor cells (<5% total tumor cells) and/or were present within granulocytes (Fig. 3c) in areas of inflammation or in non-identifiable cells. The number of granulocytes or non-identifiable cells stained was often equal to or greater than the number of positive tumor cells.

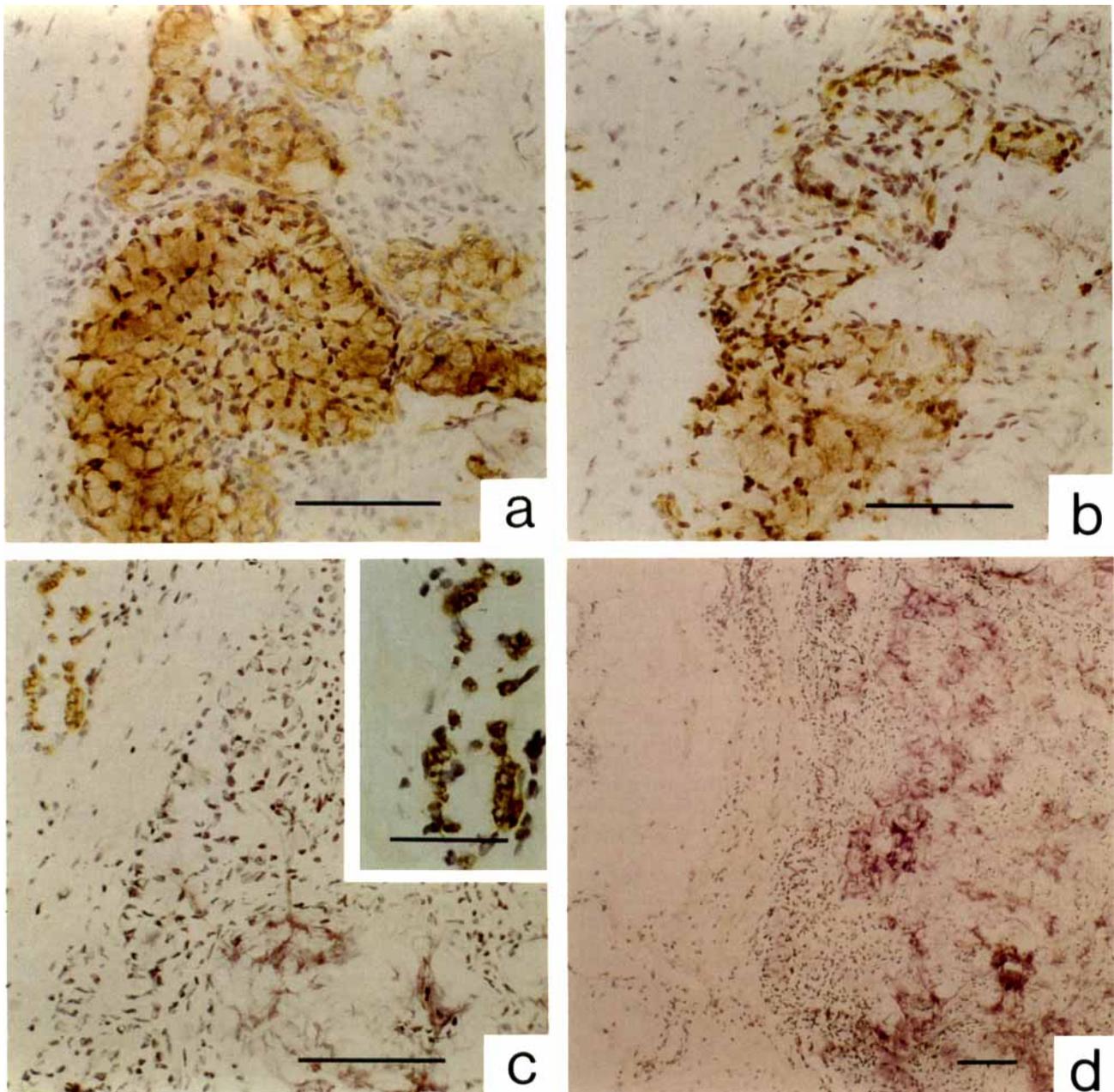
#### DISCUSSION

Using a computer program specifically written for this purpose (Lucas *et al.*, 1991), we have synthesized and tested oligonucleotide primer sets against individual CEA-related cDNA clones that specifically amplify CEA, NCA, CGM6 (NCA-95), BGP, CGM1, CGM7 transcripts and a set that will amplify only members of the PSG sub-group. For other members of the CEA-gene family (CGM2, CGM8, 9, 10 and 11), no transcripts have so far been described. Furthermore, sequence analyses indicate that these members, apart from CGM2, contain stop codons and apparently represent pseudogenes (Khan *et al.*, 1992). In future, the possibility of analyzing the expression of different splice products from individual genes, *e.g.*, BGP or individual PSGs (Thompson *et al.*, 1991), may also be taken into consideration in the design of new oligonucleotide primers. Furthermore, we are presently developing a CGM2-specific primer set in the search for transcripts from this gene.

The results gained in the analysis of selected tumors located in the brain revealed no CEA-related transcripts in neuro-epithelial tumors nor in mesenchymal tumors. On the other

hand, a poorly differentiated adenocarcinoma, which represents a primary nasal-sinus tumor, was shown to co-express CEA and NCA transcripts. These data support immunohistochemical studies carried out by Ang *et al.* (1990), who were unable to find CEA in 19 different brain primary tumors, whereas 6 brain metastases of primary tumors in the gastrointestinal tract were positive. Our results indicate that the sensitivity level of the RT/PCR is acceptable, in that the expected positivity and negativity based on published immunohistochemical data is reproduced.

Co-expression of NCA and CEA transcripts in ovarian mucinous adenocarcinomas was confirmed using MAbs specific against CEA and NCA-50/90. Other immunohistochemical investigations into the expression of CEA and NCA in ovarian tumors correlate well with our data (Motoyama *et al.*, 1990). In brief, these investigations also identified CEA and NCA in all ovarian mucinous tumors. CEA and NCA were also present in a few endometrioid and clear-cell carcinomas, but rarely in serous ovarian carcinomas, although only a small percentage of tumor cells were positive. Co-expression of CEA and NCA is often found in other types of adenocarcinomas, *e.g.*, colonic adenocarcinomas, as shown by DNA/RNA hybridization analyses (Chi *et al.*, 1991). The immunohistochemical staining on serial sections has often, but not always, revealed that the same tumor cells express both CEA and NCA. It appears that co-regulation of gene expression may exist in such cases. The genes encoding CEA and NCA are directly adjacent on the long arm of chromosome 19, being separated by only approximately 16 kb (data not shown). The coordination of expression of these genes could, therefore, be under the control of common cis-acting regulatory elements. However, in granulocytes, NCA but not CEA is expressed (Berling *et al.*, 1990), indicating that gene-specific control elements must also exist. Indeed, in these investigations, some of the tumor cells expressed CEA but not NCA, or vice versa. In a few other tumors, the CEAall primer set yielded amplification products, but the specific primer sets were all negative (Table III). This suggests either amplification of unknown CEA-gene-family



**FIGURE 3** – Identification of CEA-related antigens in an ovarian mucinous adenocarcinoma by immunohistochemical staining. Serial tumor cryosections were incubated with specific MAbs to identify: *a*, CEA (26/3/13); *b*, NCA-50/90 (9A6FR); and *c*, NCA-95 (80H3). In *d*, lower magnification of the same area is shown in the negative control without first antibody. Although serial sections were analyzed, the region shown for NCA-95 (*c*) that stains granulocytes (see inset for stronger magnification) does not correspond exactly with those for CEA and NCA, but both regions are covered in the negative control (*d*). Bars indicate 100  $\mu\text{m}$  (*a-d*), 50  $\mu\text{m}$  for inset in (*c*).

members, or more probably, co-expression of more than one known family member at low levels where individual transcripts cannot be visualized at the present sensitivity level. However, confirmation of such low expression levels would be only of academic interest.

With 200 ng of total RNA, the sensitivity of the PCR system was lower than that of the immunohistochemical method, since in the serous, clear-cell and endometrioid ovarian carcinomas, as well as one mucinous carcinoma, no CEA-related transcripts were detected. The PCR conditions were then modified

by increasing the amount of total RNA to 1  $\mu\text{g}$ . Under these conditions, the immunochemical and PCR analyses yielded more comparable results for expression of CEA-gene-family members. However, the focal staining seen in some tumor cells for NCA-50/90 and NCA-95 was still not detected with the PCR system. Although the PCR sensitivity may easily be further increased, problems could then arise with amplification of NCA, CGM1, CGM6, CGM7 and BGP transcripts present in granulocytes/leukocytes (Berling *et al.*, 1990; Kuroki *et al.*, 1991) that would lead to false positives. Indeed, the

TABLE IV - COMPARISON OF RT/PCR-ASSAY WITH IMMUNOHISTOCHEMICAL DETECTION OF CEA-GENE-FAMILY MEMBERS IN OVARIAN TUMORS

Tumor	Immunohistochemistry			RT/PCR assay		
	CEA	NCA-50/90	NCA-95	CEA	NCA	CGM6
Mucinous	(+)	+	-	+	+	-
Mucinous	(+)	+	-	+	+	-
Mucinous	+	+	-	+	+	-
Mucinous	+	+	-	+	+	-
Mucinous	+	+	-	+	+	-
Serous	-	(+)	-	(+)	(+)	-
Serous	-	(+)	(+)	-	-	-
Serous	-	(+)	(+)	-	-	-
Serous	-	-	-	-	-	-
Serous	-	-	-	-	-	-
Endometrioid	-	-	-	-	-	-
Endometrioid	-	-	-	-	-	-
Endometrioid	(+)	(+)	-	+	-	-
Endometrioid	-	(+)	(+)	-	(+)	-
Clear cell	-	(+)	(+)	+	+	-
Clear cell	-	(+)	(+)	-	-	-
Clear cell	-	-	-	-	-	-
Clear cell	-	(+)	-	-	-	-

(+) indicates only a weak signal in the RT/PCR assay or only  $\leq 5\%$  of the tumor cells stained positively.

number of granulocytes/leukocytes visualized in those tumors showing positivity only after immunohistochemical staining was often equal to or greater than the number of NCA-50/90- or NCA-95-positive tumor cells. However, in the present PCR conditions, this has not been a problem; and in those tumors where NCA transcripts were found, immunohistochemical analysis confirmed that the tumor cells themselves were NCA-50/90-positive. Even without the latter control, where NCA products are found in the absence of amplification products for CGM6, as in all the tumors investigated here, it may be concluded that NCA is, indeed, expressed in the tumor cells and not in granulocytes, since steady-state CGM6 mRNA levels are generally higher than NCA mRNA levels in granulocytes (Berling *et al.*, 1990).

In general, this RT/PCR assay provides a useful system for specific identification of transcripts for each CEA-related gene. This cannot be achieved at the antigen level for all family members, due to the lack of specific antibodies for each species, or through DNA/RNA hybridization studies, because many probes will cross-hybridize or be of limited sensitivity. A further advantage of this PCR system is that its unlimited sen-

sitivity makes it feasible to carry out such studies on biopsy material (*e.g.*, fine-needle aspirates) where the amounts of tumor tissue available for analysis are very small. In such cases, higher sensitivity may be attained by increasing the number of cycles rather than the RNA amounts, which will also be limiting.

The preliminary studies presented here indicate that not only CEA, but also NCA, may be a useful tumor marker for ovarian mucinous adenocarcinomas, whereas the expression of other CEA-family members is more limited or completely absent from the tumors investigated so far. These analyses will be continued and further optimized on other tumors in which expression of CEA and CEA-related antigens have been reported, *e.g.*, tumors of the breast, lung and pancreas, and trophoblastic tumors.

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