[CANCER RESEARCH 48, 2550–2554, May 1, 1988]

Chromosomal Localization of the Carcinoembryonic Antigen Gene Family and Differential Expression in Various Tumors

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ABSTRACT

Carcinoembryonic antigen (CEA) is a glycoprotein which is important as a tumor marker for a number of human cancers. It is a member of a gene family comprising about 10 closely related genes. In order to characterize mRNAs transcribed from individual genes we have identified by DNA and RNA hybridization experiments, gene-specific sequences from the 3′ noncoding regions of CEA, and of nonspecific cross-reacting antigen (NCA) mRNAs, which have been recently cloned. With these probes, CEA mRNAs with lengths of 3.5 and 3.0 kilobases and an NCA mRNA species of 2.5 kilobases were identified in various human tumors. A 2.2-kilobase mRNA species, however, could only be detected in leukocytes of patients with chronic myeloid leukemia by hybridization with a probe from the immunoglobulin-like repeat domain of CEA. This region is known to be very similar among the various members of the CEA gene family, and indeed the probe hybridizes with all four mRNA species. *In situ* hybridization with a cross-hybridizing probe from the NCA gene localized the members of the CEA gene family to the short and to the long arm of chromosome 19. In addition, a CEA cDNA probe was found to hybridize to the long arm of chromosome 19 only.

INTRODUCTION

CEA,[2] which was originally discovered by Gold and Freedman (1), is probably the most widespread marker in tumors of epithelial origin, especially in colon tumors (2). However, a number of closely related molecules have been discovered using immunological methods (2, 3), and more recently, a family of about 10 genes was found to exist (4, 5). This complication, along with the fact that CEA is also present in normal tissues (6), put the reliability of CEA as a tumor marker in question. With the advent of monoclonal antibodies, the specific identification of individual members of the CEA family has been partially realized (7). Recently, the primary sequence for CEA was reported (5, 8, 9), as well as that of a nonspecific cross-reacting antigen (NCA) (4, 10). This information should eventually help to identify unique epitopes for specific molecules, if they exist. In any case, differences in the 3′ untranslated regions of the mature mRNA molecules for CEA and NCA have been found, whereas the coding regions appear to be highly conserved. In this paper, we have utilized this knowledge in identifying CEA, as well as NCA mRNAs through Northern analyses in a number of different tumors. Finally, the chromosomal location of this gene family has been determined.

MATERIALS AND METHODS

Tissues. Leukocytes from CML patients were isolated from EDTA-anticoagulated peripheral venous blood, after dextran sedimentation of erythrocytes, and further purified by centrifugation through 0.9 g/ml Percoll (Pharmacia) in PBS at 1800 × *g*, for 40 min. Cells were washed in PBS and frozen in liquid nitrogen. Human tumors had been propagated and amplified in athymic mice (11). Tumor tissues were separated from normal and necrotic tissue, washed in cold PBS, frozen in liquid nitrogen and stored at −140°C.

Isolation and Sequence Analysis of pCEA3. For the isolation of the CEA cDNA clone pCEA3, a cDNA library was used, which had been prepared from human colon tumor mRNA (8). The library was screened under stringent hybridization conditions with the 534-base pair *Pst*I fragment of pCEA1 comprising part of repeats RII and RIII (the former repeats a and b) of CEA cDNA as described before (8). For sequencing, suitable subfragments of pCEA3 were cloned into M13 vectors and sequenced according to Sanger (12). Regions of pCEA3, overlapping with previously characterized CEA cDNA clones (8) were sequenced to an extent which was sufficient to prove that pCEA3 was derived from CEA mRNA.

Isolation and Southern Analysis of Genomic DNA. DNA was isolated from human peripheral leukocytes. Fresh blood was centrifuged at 120 × *g* for 5 min at 2°C. The white blood cells of the upper layer were collected and digested with 100 μg/ml of proteinase K in 50 mM Tris-Cl (pH 7.5), 20 mM EDTA (pH 8.0), 0.1% SDS at 37°C over night. After phenol/chloroform (1:1) extraction and ethanol precipitation, the high molecular weight DNA was resuspended in 50 mM Tris-Cl (pH 7.5), 20 mM EDTA (pH 8.0) and banded after addition of 1.25 g cesium chloride per ml by centrifugation at 136,000 × *g* for 48 h. The DNA containing fractions of the gradient were extensively dialyzed against 10 mM Tris-Cl (pH 7.5), 1 mM EDTA. For Southern blot analysis, 2.5 μg of DNA per sample were cut with a restriction endonuclease and electrophoresed in 0.8% agarose using a minigel apparatus (LKB). The DNA fragments were transferred to charged nylon membranes (GeneScreenPlus, New England Nuclear). The blots were prehybridized in 40% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate at 42°C for 15–30 min. Then 100 μg of sonicated, heat-denatured, salmon sperm DNA together with 5 × 10^5 dpm of ^{32}P-labeled cDNA probes (13) per ml (specific radioactivity, 6–8 × 10^8 dpm/μg) were added and hybridization was carried out at 42°C over night. Filters were washed twice each in 2xSSPE (1xSSPE = 0.18 M NaCl, 10 mM sodium phosphate (pH 7.4), 1 mM EDTA) at room temperature for 15 min, in 2xSSPE, 1% SDS at 65°C for 30 min and in 0.1xSSPE at room temperature for 5 min. The filters were briefly blotted on Whatman 3MM paper and exposed to X-ray film (Kodak X-Omat AR) at −70°C in the presence of an intensifying screen. For high stringency conditions the filters were rewashed twice in 0.1xSSPE, 1% SDS at 65°C for 30 min.

RNA Analysis by Northern Blot Hybridization. Total RNA was extracted from leukocytes and tumor tissues, pulverized in liquid nitrogen, by the guanidinium thiocyanate method as described by Fiddes & Goodman (14). The yield of total RNA was between 0.1–1 mg per g of tissue. Poly(A)$^+$ RNA was isolated by two rounds of chromatography on oligo(dT) cellulose (Sigma) according to Aviv and Leder (15). For Northern blot hybridization, RNAs were electrophoresed on a denaturing 1% agarose gel containing 10 mM methylmercury hydroxide, and transferred to charged nylon membranes according to Alwine *et al.* (16). *Hind*III-digested λ DNA was included as a size marker. Prehybridization was performed in 50% formamide, 1% SDS, 10% dextran sulfate, 1 M NaCl at 42°C for 6 h. The blots were hybridized with [^{32}P]-cDNA probes in the same buffer after addition of 100 μg/ml denatured

Received 9/29/87; revised 1/21/88; accepted 1/29/88.

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[2] The abbreviations used are: CEA, carcinoembryonic antigen; NCA, nonspecific cross-reacting antigen; CML, chronic myeloid leukemia; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

salmon sperm DNA at 42°C for 12–20 h. After hybridization, the filters were washed in 2xSSPE at room temperature, then in 2xSSPE, 1% SDS at 60°C for 30 min and again in 2xSSPE at room temperature. In some experiments the filters were washed after autoradiography under stringent conditions in 0.1xSSPE, 0.1% SDS for 30 min at 65°C.

***In Situ* Hybridization.** Chromosome preparations were made from peripheral lymphocytes of normal human males. A detailed description of the culture conditions and the BrdU treatment has been given elsewhere (17). *In situ* hybridization of chromosome spreads was done according to Harper and Saunders (18). In brief, the denatured chromosome preparations were hybridized in 50% formamide, 10% dextran sulfate, and 2xSSC (0.3 M NaCl, 0.034 M Na-citrate) for 18 h at 37°C, with [^{3}H]dATP- [^{3}H]dCTP- and [^{3}H]TTP-labeled probes. A specific activity of about 6–8 × 10^7 cpm/μg DNA was obtained by using the random priming technique of Feinberg and Vogelstein (13, 19). Following hybridization, the slides were rinsed thoroughly in 50% formamide/2xSSC at 39°C and coated with Kodak NTB 2 nuclear track emulsion. After 5–8 days of exposure, the chromosomes were R-banded by the method of Perry and Wolff (20).

RESULTS

Specificity of CEA and NCA cDNA Probes at the Genomic DNA Level. In order to study the expression of single members of the CEA gene family in tumors, gene specific probes are needed. Probes derived from the coding region of CEA and NCA cDNA clones are not suitable, because of the high degree of homology [up to 93% at the nucleotide level (10)]. In contrast, comparison of the nucleotide sequence of the 3′ noncoding regions of CEA and NCA mRNA reveals no homology except for a stretch of 40 nucleotides directly downstream from the stop codon (8, 10). The previously isolated CEA cDNA clone pCEA2, however, contains only a small part of the 3′ noncoding region of CEA mRNA (8). We therefore isolated pCEA3 which extends about 450-base pair further downstream (Fig. 1). Sequence analysis revealed a few minor, however unequivocal differences to the nucleotide sequence of CEA cDNA presented by Oikawa *et al.* (5). Assuming the numbering from these authors, the following differences should be noted: Several differences are clustered around the 3′ end of the Alu sequence present in the 3′ noncoding region of CEA mRNA (5, 8). In our CEA cDNA clone pCEA3 at position 2433 a G is found instead of a T and the A in position 2439 is missing, whereas at position 2451 two additional As are present. By the latter difference the length of the direct repeat flanking the Alu sequence, is increased from 12 to 16 nucleotides.

As potential probes specific for CEA mRNA, we selected a 195-base pair *Ssp*I fragment (CEA 3′) and for some experiments, a 408-base pair *Rsa*I/*Pst*I fragment (CEA 3′a) of the CEA cDNA clone pCEA3, downstream from the Alu-type repetitive sequence. For the detection of NCA mRNA we chose a 1.5-kilobase *Eco*RI fragment (NCA 3′) from the NCA cDNA clone 9 which spans nearly the whole of the 3′ noncoding region of NCA mRNA (10). The location of these probes is depicted in Fig. 1. The specificity of the probes derived from the 3′ noncoding regions of CEA and NCA cDNA was assessed by blot hybridizations with genomic DNA and RNA. With both probes only one DNA fragment hybridized from human genomic DNA digested either with *Eco*RI or *Sst*I under stringent conditions (Fig. 2, *B* and *C*, *lanes 6*, *8*, *10*, and *12*). Upon hybridization, under more relaxed conditions, the CEA 3′ probe revealed only one additional band indicating that this sequence is present in one or two copies per haploid genome (Fig. 2*C*, *lanes 9* and *11*). In contrast, the sequence from the 3′ untranslated region of NCA cDNA hybridized under identical conditions with multiple *Eco*RI and *Sst*I fragments (Fig. 2*B*, *lanes 5* and *7*). This result indicates the presence of several genes which share related 3′ noncoding regions. A probe from the coding region comprising part of the immunoglobulin-like domain of CEA cDNA (Fig. 1, *repeat*) also reveals multiple bands (Fig. 2*A*). However, in contrast to the NCA 3′ probe most of these hybrids are also stable under stringent conditions. This is in agreement with the finding that the translated region of different genes of the CEA family are closely related (10).

Specificity of CEA and NCA cDNA Probes at the RNA Level. The usefulness of these probes for the identification of CEA and NCA mRNA and mRNAs transcribed from other members of the CEA gene family was evaluated by RNA blot hybridization experiments. We first used the probe derived from the coding region of CEA mRNA, employing moderately stringent hybridization conditions to be able to detect all expressed members of the CEA gene family. We chose RNA from a colonic tumor and leukocytes from CML patients because these tissues or cells are known to express CEA and NCA, respectively (1, 21). RNA from the colon tumor was found to contain three mRNA species with lengths of 3.5, 3.0, and 2.5 kilobases. Under the same experimental conditions in CML RNA a 2.5- and a 2.2-kilobase mRNA species could be identified (Fig. 3*A*, *lanes 1* and *2*). In this latter case, four separate CML leukocyte RNA preparations were investigated. One preparation origi-

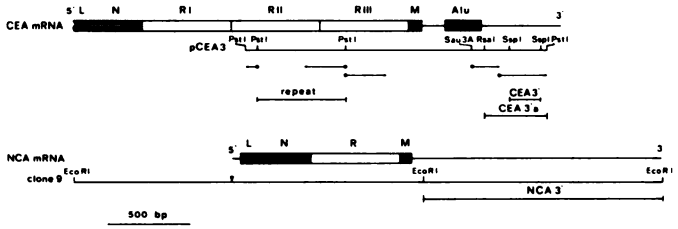

Fig. 1. Structure of CEA and an NCA mRNA and location of cDNA probes. A graphic representation of CEA (5) and NCA mRNA (10) is shown. Homologous coding regions are depicted by similar shading. The coding regions are composed of a leader peptide (*L*), an N-terminal region (*N*), immunoglobulin-like repeated domains (R, RI, RII, RIII) and a putative membrane anchoring-domain (*M*). ***Box in the 3′ untranslated region of CEA mRNA***, truncated Alu sequence (5, 8). Below, the length and location of CEA cDNA clone pCEA3 and NCA cDNA clone 9 are indicated (10). The *Pst*I and *Eco*RI sites flanking the cDNA fragments are artificially created by the cloning procedure. *Arrows*, location and extent of sequencing. Only the *Sau*3A restriction endonuclease site relevant for sequencing is shown. The cDNA fragments used as probes in experiments described in this paper are shown below the cDNA clones. *Arrowhead*, position where an unrelated cDNA was fused to the NCA cDNA during cloning (10).

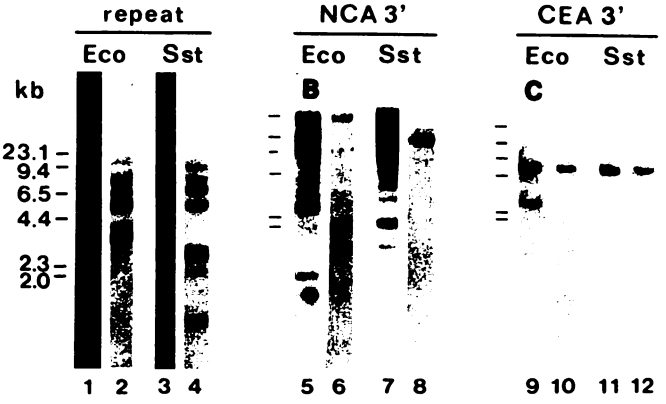

Fig. 2. Characterization of CEA and NCA cDNA probes by hybridization with human genomic DNA. Genomic DNA was digested either with *Eco*RI or *Sst*I, size fractionated on agarose gels, transferred to a nylon membrane, and hybridized with cDNA probes derived from: *A*, the immunoglobulin-like region of CEA mRNA (*repeat*); *B*, the 3′ untranslated region of an NCA mRNA (*NCA 3′*); or *C*, CEA cDNA (*CEA 3′*). The filters were first washed under moderately stringent conditions (*lanes with odd numbers*), then, after autoradiography, rewashed under stringent conditions (*lanes with even numbers*).

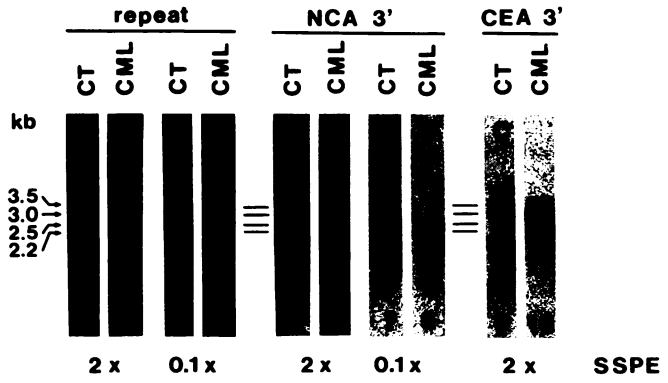

Fig. 3. Characterization of CEA and NCA cDNA probes by Northern blot hybridizations. 2 μg of poly(A)$^+$ RNA from a colonic adenocarcinoma (*CT*) or 10 μg of total RNA from leukocytes of a CML patient were size fractionated on denaturing agarose gels, blotted onto nylon membranes, and hybridized with cDNA probes derived from: *A*, the immunoglobulin-like region of CEA mRNA (*repeat*); *B*, the 3′ untranslated region of an NCA mRNA (*NCA 3′*); or *C*, CEA cDNA (*CEA 3′*). The same RNA blot was used for hybridization with the various probes after decay of the ^{32}P-radioactivity of the original probes. The filters were washed under moderately stringent (2xSSPE) and highly stringent condition (0.1xSSPE) as described in Fig. 2, *legend*. The apparent RNA bands with a size less than 2 kilobases visible in most lanes are assumed to be an artefact caused by compression of degraded RNA by the 18 S rRNA which migrates just above these regions.

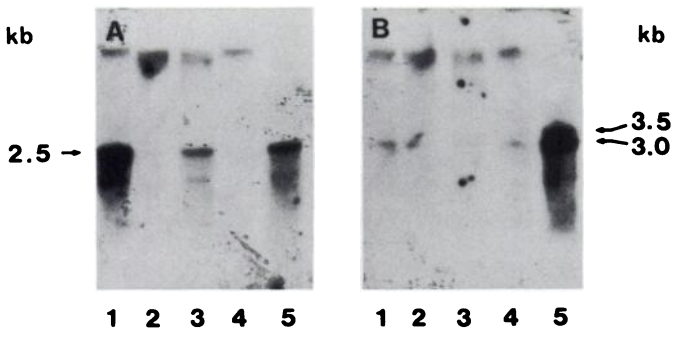

Fig. 4. Expression of CEA and NCA mRNA in human tumors. 10 μg of total RNA from an adenocarcinoma of the pancreas (*lane 1*), a squamous carcinoma of the cervix (*lane 2*) or of the lung (*lane 3*), a gall bladder carcinoma (*lane 4*), and a colonic adenocarcinoma (*lane 5*) were size fractionated on a denaturing agarose gel, blotted onto a nylon membrane, and hybridized with cDNA probes from the 3′ untranslated region of an NCA mRNA (*A*) or with the 408-base pair *Rsa*/*Pst*I fragment of CEA cDNA (*CEA 3′a* in Fig. 1) (*B*). The filters were washed at 60°C in 2xSSPE and autoradiographed. For explanation of the smaller bands see Fig. 3, *legend*.

nated from a single patient and the others were pooled from two to nine patients. The hybridization patterns were identical for all four preparations. Additional bands with a smaller size seen in most lanes will not be considered here in more detail due to their weak hybridization signals and possible artifactual origin (see *legends* to Figs. 3 and 4). After a stringent wash of the RNA blots, only the hybrids of the 3.5- and 3.0-kilobase mRNA species were stable (Fig. 3*A*, *lanes 3* and *4*). Further, only these two mRNAs were found to hybridize with the CEA 3′ probe in colon tumor RNA both under relaxed (Fig. 3*C*, *lanes 9* and *10*) and stringent hybridization conditions (data not shown). Even after prolonged exposure of the filters no additional bands could be detected. With the probe from the NCA mRNA 3′ noncoding region in colon tumor and CML RNA a single 2.5-kilobase mRNA species could be identified irrespective of the washing condition used (Fig. 3*B*, *lanes 5–8*). These results suggest, that the probes excised from the 3′ untranslated regions of CEA and NCA cDNA are specific for CEA and NCA mRNA respectively.

Expression of CEA and NCA mRNAs in Tumors. The above-mentioned probes were also used to screen a panel of human solid tumors propagated in nude mice for the expression of CEA/NCA mRNAs. However, the 195-base pair *Ssp*I fragment (CEA 3′ in Fig. 1) from the 3′ untranslated region of CEA cDNA was replaced by a 408-base pair *Rsa*I/*Pst*I fragment from the same region (CEA 3′a in Fig. 1). This probe was as specific as the smaller fragment for the detection of CEA mRNA, but yielded stronger hybridization signals (data not shown). CEA mRNAs were found in adenocarcinomas of the colon and, to a lower extent, in an adenocarcinoma of the pancreas, as well as in a carcinoma of the gall bladder (Fig. 4*B*). The colon and the pancreas adenocarcinomas expressed NCA mRNA in addition to CEA mRNA. In a squamous carcinoma of the lung, only NCA mRNA could be detected (Fig. 4*A*). The variation in the relative and absolute amounts of CEA and NCA mRNA among different tumors was very pronounced. With the probe from the repeat domain of CEA cDNA, no additional mRNA species could be detected (not shown). The three probes used in this experiment did not hybridize with any mRNA species when RNA from a squamous carcinoma of the cervix, a hypernephroma of the kidney, an adenocarcinoma of the stomach and of the breast, an embryonic carcinoma of the testis, a large cell carcinoma of the lung, a melanoma, an osteosarcoma or a mesothelioma of the pleura were tested (data not shown). In addition, all four colonic adenocarcinomas assayed directly after resection were found to contain both CEA and NCA mRNAs (data not shown).

Chromosomal Localization of the CEA Gene Family. A 1.3-kilobase *Eco*RI DNA fragment from the NCA gene, which besides intron sequences, contains the exon for the N-terminal half of the repeat domain,[3] has been localized by *in situ* hybridization to chromosome 19. 610 silver grains were scored on 45 metaphases with 131 (21.5%) grains present on chromosome 19 (Fig. 5*A*). Fine mapping of the grain distribution over chromosome 19 revealed two specific hybridization sites with a major grain accumulation over 19q31-q32 (53/131) and a minor signal over 19p13.2-p13.3 (21/131) (Fig. 5, *B* and *C*). Furthermore, *in situ* hybridization with the 534-base pair *Pst*I fragment from the repeat region of the CEA cDNA clone pCEA1 (8), revealed a corresponding grain accumulation over chromosome 19 (56 out of 388 grains scored on 71 metaphases) (Fig. 6*A*). However, in contrast to the probe from the NCA gene, only one specific hybridization site was localized in 19q31-q32 (21/56) (Fig. 6*B*).

DISCUSSION

Probes derived from the 3′ untranslated region of CEA and NCA cDNAs appear to be gene specific when stringent hybridization conditions are used (see Figs. 2 and 3). While sequences related to the 3′ noncoding region of CEA mRNA are present in only one or two copies per haploid genome, regions related to the 3′ noncoding sequence of the here characterized NCA mRNA seem to be shared by several members of the CEA gene family. This notion is supported by the fact that human genomic clones representing three different genomic loci hybridize with both probes from the coding region of CEA cDNA and the 3′ untranslated region of the NCA cDNA.[4] However, the degree of relatedness of NCA-like, 3′ untranslated sequences is lower than the similarity between coding regions of the CEA gene family. This is indicated by the instability of hybrids between the probes from the 3′ noncoding region of NCA cDNA and

[3] Unpublished results.
[4] J. A. Thompson, unpublished results.

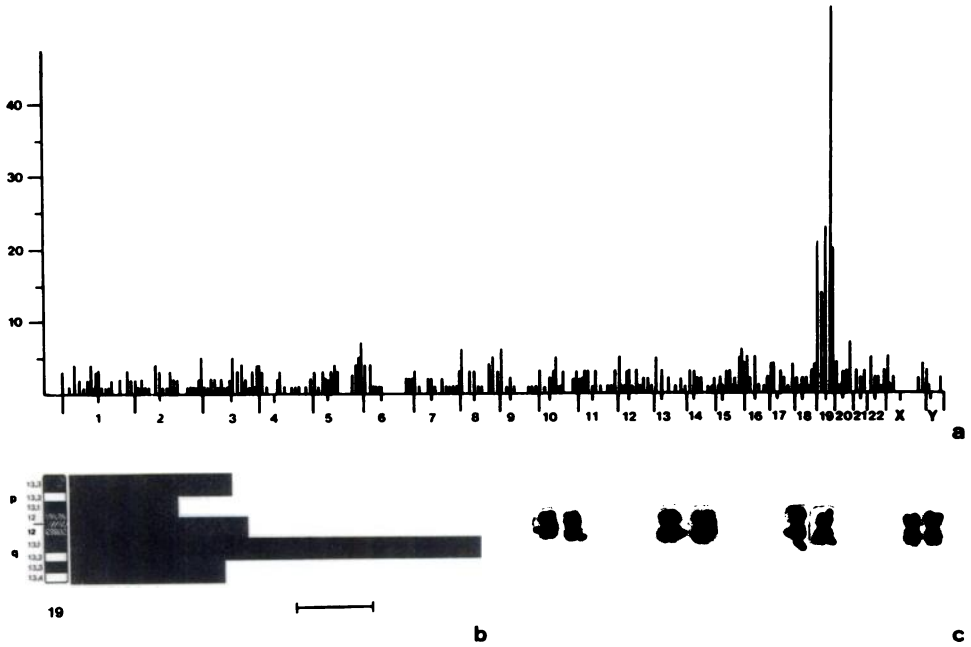

Fig. 5. *In situ* hybridization with a genomic NCA probe. For hybridization, the genomic 1.3-kilobase *Eco*RI DNA fragment from λ39.2 (4) was used. *A*, histogram showing the grain distribution in 45 metaphase cells. Of 610 total grains, 131 (21.5%) are located on chromosome 19; *bar*, 10 grains. The diagram of the grain distribution over chromosome 19 localizes the CEA gene family to 19p13.2-p13.3 and to 19q31-q32 (*B*). *C*, four specifically labeled pairs of chromosome 19. Note the simultaneous labeling of the short and the long arm in some chromosomes 19.

Fig. 6. *In situ* hybridization with a CEA cDNA probe. The 534-base pair *Pst*I fragment from the CEA cDNA clone pCEA1 (8) was used for hybridization. 388 grains were scored on 71 metaphase spreads with 56 grains (14.4%) over chromosome 19 (*A*). The diagram shows the specific grain localization on 19q31-q32; *bar*, 10 grains (*B*).

the 3′ noncoding region of other members of the gene family under stringent hybridization conditions (Fig. 2*B*). The uniqueness of the 3′ noncoding region of CEA mRNA beyond 40 nucleotides of the stop codon (9) and the presence of multiple, related copies of the 3′ noncoding exon(s) of the NCA gene in the human genome imply that relatively late in evolution, an additional exon may have been inserted into the originally "NCA-like" 3′ untranslated exon of the CEA gene. A similar event might have caused the abrupt divergence in the 3′ untranslated region of preprocathepsin B mRNA in rat and mouse (22).

With the thus characterized probes, four discrete mRNA species could be identified in different human tissues and cells by RNA blot experiments. Two mRNA species with lengths of 3.5 and 3.0 kilobases represent transcription products of the CEA gene because they hybridize with CEA cDNA probes from the immunoglobulin-like domain and the 3′ noncoding region under stringent hybridization conditions. The difference in length is probably caused by the alternative use of two polyadenylation signals present in the 3′ noncoding region of CEA mRNA (5). The absolute size and the size difference of the CEA mRNAs is in good agreement with published sequence data of CEA cDNAs (5). By stringent hybridization with the probe from the 3′ untranslated region of an NCA cDNA we have identified a single 2.5-kilobase mRNA species. At present it is not known whether this mRNA species codes for NCA-55 or NCA-95 or both because these antigens might differ only in their degree of glycosylation (10). In addition, a 2.2-kilobase mRNA species was found by hybridization with the probe coding for the immunoglobulin-like domain of CEA under nonstringent conditions. This latter species does not hybridize with probes from the 3′ untranslated region of CEA or NCA cDNA, and thus represents an mRNA derived from a yet unknown member of the CEA gene family.

In an initial screening, CEA and NCA mRNAs were found in tumors derived from various organs and tissues but showed variability in their absolute and relative amounts. Strong expression of CEA mRNA was found in adenocarcinomas of the colon and to a lesser extent in an adenocarcinoma of the pancreas. This agrees well with the high CEA positivity rate of these tumors (2). So far, the 2.2-kilobase mRNA species has been detected only in CML cells, and may therefore represent the transcription product of a member of the CEA gene family which is specifically expressed in the myeloid lineage. In CML cells, two NCA species with molecular weights of 97,000 and 160,000 have been identified by immunological methods.[5] To be able to assign the two proteins to the 2.2-kilobase and 2.5-kilobase mRNA species found in CML cells, the cloning of the 2.2-kilobase mRNA species and partial sequencing of the proteins will have to be accomplished. In future, extended screenings of human tumors and corresponding normal tissues for the presence of mRNAs of members of the CEA gene family might reveal a better correlation between the expression of a certain member of the CEA family and the malignant phenotype.

In order to determine the chromosomal localization of the different members of the CEA gene family, we chose probes from the NCA gene and a CEA cDNA, which both contain sequences from the repeat region. This region appears to represent a basic building unit and cross-hybridizes with other members of the CEA gene family (see Fig. 2*A*).[3] The localization of all genes belonging to this family to chromosome 19 is

[5] R. Bhardwaj, personal communication.

in agreement with the findings of Kamarck *et al.* who also used a CEA cDNA fragment from the same region, to probe the DNA extracted from a panel of mouse/human somatic cell hybrids (9). A major gene cluster is found on the long arm of chromosome 19, and a second locus is indicated at the end of the short arm which possibly contains the NCA gene. As the high stringency hybridization conditions used for Southern and Northern analyses cannot be applied here, the chromosomal localization of specific genes cannot be unequivocally determined so far. We are at present involved in a more detailed characterization of these loci by chromosome walking using a cosmid library.

ACKNOWLEDGMENTS

We would like to thank Ute Dentler for the propagation of tumors in nude mice and Martina Weiss for excellent technical assistance. We gratefully acknowledge the support of a grant by the Dr. Mildred Scheel Stiftung für Krebsforschung.

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