A Complete Complementary DNA for the Oncodevelopmental Calcium-binding Protein, Oncomodulin*

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RNA from a rat liver tumor (Morris hepatoma 5123tc) was used to construct cDNAs together comprising the complete coding sequence of rat oncomodulin mRNA. Information obtained from these cDNAs as well as from primer extension analysis gave a deduced length for the complete oncomodulin mRNA of approximately 680 nucleotides (excluding the poly(A) tail) including a 5' untranslated region of 97 ± 2 nucleotides, a 324-nucleotide-coding sequence and a 259-nucleotide 3' non-coding region. Comparison of the oncomodulin cDNA sequence with those coding for other members of the calcium-binding protein family shows little homology with the exception of a recently reported parvalbumin cDNA where the oncomodulin and parvalbumin nucleotide sequences are 59% identical in the protein-coding region. RNA blot analysis of poly(A) RNA from normal adult rat liver gave no evidence of oncomodulin expression in this tissue. A single RNA species was detected, however, in RNA extracts from the hepatoma and from rat and human placenta. A probe prepared from one of the rat oncomodulin cDNAs hybridized with a single DNA species in restriction digests of hepatoma and normal DNA from rat and sequences in DNA of humans and other mammals. A 38-nucleotide sequence spanning the 5'-untranslated region and the first seven codons of the oncomodulin cDNA was far less homologous than was the same region of a parvalbumin cDNA, to a chicken calmodulin cDNA sequence coding for the first calcium-binding domain. The oncomodulin gene appears to have diverged more from that of calmodulin than has the parvalbumin gene.

The multigene family of vertebrate calcium-binding proteins includes among others: calmodulin, parvalbumin, S-100 protein, and oncomodulin (1-4). Calmodulin occurs in all tissues of vertebrates (3). Parvalbumin is more restricted, occurring mainly in fast twitch muscles (5) but also in bone, skin, and teeth (6). S-100 protein is even more restricted, and parvalbumin nucleotide sequences are 59% identical in the protein-coding region. RNA blot analysis of poly(A) RNA from normal adult rat liver gave no evidence of oncomodulin expression in this tissue. A single RNA species was detected, however, in RNA extracts from the hepatoma and from rat and human placenta. A probe prepared from one of the rat oncomodulin cDNAs hybridized with a single DNA species in restriction digests of hepatoma and normal DNA from rat and sequences in DNA of humans and other mammals. A 38-nucleotide sequence spanning the 5'-untranslated region and the first seven codons of the oncomodulin cDNA, was far less homologous than was the same region of a parvalbumin cDNA, to a chicken calmodulin cDNA sequence coding for the first calcium-binding domain. The oncomodulin gene appears to have diverged more from that of calmodulin than has the parvalbumin gene.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J02705.

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EXPERIMENTAL PROCEDURES

Materials—All restriction endonucleases were obtained from Bethesda Research Laboratories (BRL). Reverse transcriptase, DNA polymerase I, T, DNA ligase and RNase H were also purchased from BRL. [γ-32P]ATP was from ICN Biomedicals (Irvine, CA) and [α-32P]dNTPs were obtained from either Amersham Corp. or ICN Biomedicals. T, polynucleotide kinase was purchased from New England Biolabs (Madison, WI) and oligo(dT)-cellulose was obtained from BRL. Biodyne nylon membranes were from ICN Biomedicals. All oligomers used in the present study were synthesized on an Applied Biosystems (Foster City, CA) automatic DNA synthesizer. The vector M13mp9 and the 17-mer sequencing primer were obtained from Pharmacia. Formamid used in hybridizations was nuclear acid grade from BRL. All other chemicals were reagent grade.

RNA Extraction and Fractionation—Total RNA was extracted from adult Sprague-Dawley rat liver tissue, Morris hepatoma 5123tc carried in Buffalo rats, or human placenta and choriocarcinoma tissues (14), using the procedure of Chirgwin et al. (19). Tissue samples were used fresh and homogenized with a Kinematica (Brinkman Instruments) homogenizer at full speed for 60 s. The crude RNA was further purified by centrifugation through a cushion of CsCl (20).
Poly(A) RNA was isolated from total RNA by oligo(dT)-cellulose chromatography (21, 22).

Construction and Screening of a cDNA Library—Poly(A) RNA from Morris hepatoma was used to construct double-stranded cDNA according to the procedure of Gubler and Hoffman (29) with some minor modifications. The double-stranded cDNA was inserted into the HincII site of M13mp9 by blunt-end ligation, and the recombinant DNA was used to transform Escherichia coli JM 103 according to the procedure of Hanahan (24).

A pool of 17-nucleotide synthetic DNAs, complementary to all the possible sequences corresponding to oncomodulin amino acids 101–106, was used to probe the cDNA library as described by Woods (25). A positive plaque (clone 22-1) was identified by autoradiography, plaque purification, and phage DNA was isolated according to Sanger et al. (26).

Primer Extension and Cloning of the 5'-End of the Message—Primer extension analysis was performed essentially as described by Devine et al. (27). Three different single-stranded DNA primers were used: the first two were derived from restriction fragments of the original incomplete cDNA clone (see legend to Fig. 1) and the third was a synthetic oligonucleotide corresponding to nucleotides 203–222. Fifteen to twenty μg of rat hepatoma total RNA were hybridized overnight in sealed capillaries with an excess of primer at 60 °C with the first two primers (62 or 66 nucleotides long) or at 50 °C with the 20-nucleotide synthetic DNA primer. The products of the extension reactions were analyzed on 8% acrylamide gels.

A pool of cDNA clones (pONCO) corresponding to the 5'-end of the oncomodulin message was prepared beginning with hepatoma total RNA size fractionated on a 6–35% sucrose gradient and a 20-nucleotide synthetic DNA primer. Ten μg of total RNA was hybridized with two pONCO primers (52 or 66 nucleotides long) or at 40 °C with the third primer (22 nucleotides long). The hybridization solution contained 50% formamide, 10 mm sodium citrate, pH 7.0, 0.5% sodium dodecyl sulfate (10 min at room temperature), and 10 mm sodium dodecyl sulfate (10 min at room temperature).

Following hybridization and washing of unbound probe, filters were blotted dry and exposed, with intensifying screens, at −80 °C for 1–4 days to Kodak SB-5 film.

RESULTS

Isolation of Oncomodulin cDNA Clones—Approximately 10,000 M13 recombinants containing cDNAs prepared from hepatoma poly(A) RNA, were screened with a pool of 16 synthetic oligonucleotides whose sequences, CT(C/T)AA(5/ A)CT(A/C)T(G/C)TACCA, are complementary to all possible sequences coding for amino acids 101–106 of the oncomodulin protein (37). One positive clone, designated 22-1, was isolated and shown by nucleotide sequence analysis to contain a 481-base pair insert consisting of a part of the oncomodulin-coding sequence, corresponding to amino acids 40–108, and a 273-base pair untranslated 3' sequence. The untranslated region includes a termination codon (TAA) and a polyadenylation signal (AATAAA) located 16 nucleotides upstream of a 14-nucleotide poly(A) tail.

A plasmid clone containing the entire 5'-coding sequence was obtained using a 20-nucleotide synthetic fragment corresponding to nucleotides 11–30 of the insert in clone 22-1 to prime the synthesis of the cDNA. The nucleotide sequence of one of the clones, pONCO, is shown in Fig. 1 together with the sequence in the insert in clone 22-1. The region of overlap between the two clones is underlined. The start codon is located at position 74 in the cloned DNA fragment. To estimate the length of the untranslated 5' sequence, primer

FIG. 1. Complete nucleotide sequence of the coding strand of the rat oncomodulin cDNA. The sequence is a composite of the data obtained from two recombinants: clone pONCO, which contains the sequence from nucleotides 1–222, and clone 22-1, which contains the sequence from nucleotides 193–659, plus 14 A residues of the poly(A) tail. The region of overlap between the two clones is underlined. Subclone 9-6, used as a probe, contains nucleotides 193–382. The DNA fragments used in primer extension analyses are: 1) a 52-nucleotide fragment derived from the AluI-HinfI restriction fragment (position 263–314), 2) a 65-nucleotide fragment derived from the BamHI-HinfI restriction fragment (position 249–314), and 3) a 20-nucleotide synthetic DNA fragment containing the sequence complementary to nucleotides 208–222.

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extension analyses were performed using various primers (see legend to Fig. 1). The results obtained with all of the three primers indicate that the total length of the 5'-noncoding-region of the oncomodulin message is 97 ± 2 nucleotides. For example, the result of a primer extension formed with the 20-nucleotide synthetic DNA primer used for the synthesis of the cDNA, is shown here in Fig. 2.

The sequence data from the various cDNA clones, along with the information obtained from the primer extension analyses, give a deduced length of approximately 680 nucleotides for oncomodulin mRNA (excluding the poly(A) tail).

Sequence analysis of clone 22-1 revealed an EcoRI restriction site within the coding sequence at the codons for amino acids 101 and 102. This site, along with a second EcoRI site located within the vector at the 3'-end of the insert, flank a sequence containing the entire 3'-untranslated region of clone 22-1. Removal of this EcoRI fragment and religation of the remainder of the clone produced a new clone (9-6) whose insert codes for oncomodulin amino acids 40-102 and contains no untranslated sequences. This clone containing only oncomodulin-coding sequence was used in all subsequent experiments for the preparation of radiolabeled probes.

RNA Blot Analyses—Northern blots probed with a radiolabeled single-stranded probe derived from clone 9-6 showed hybridization to a single RNA species of approximately 75 nucleotides in samples of RNA from rat hepatoma and rat placenta (Fig. 3a, lanes 1 and 3, respectively). This is in agreement with the deduced size of the oncomodulin mRNA (excluding the poly(A) tail) obtained from primer extension and DNA sequence analysis (see above). A single species of the same size was detected in RNA from human placenta and a choriocarcinoma (BeWo) (Fig. 3a, lanes 4 and 6, respectively).

In a separate experiment, no hybridizable RNA species was detected in normal adult rat liver even when total RNA was enriched for poly(A⁺) message and probed for oncomodulin expression (Fig. 3b, lane 4). Oncomodulin protein has not been found in normal adult rat liver (12).

DNA Blot Analyses—Restriction digests of genomic DNA from normal rat liver and from the rat hepatoma were hybridized with a single-stranded probe derived from clone 9-6. Fig. 4a shows a BamHI fragment of 2.8 kilobase pairs, an EcoRI fragment of 2.4 kilobase pairs, and a HindIII fragment of approximately 10 kilobase pairs detected by the probe in digests of DNA from normal adult rat liver. The pattern obtained with DNA from the rat hepatoma is identical both in location and in intensities of the bands (results not shown). Restriction digests of genomic DNA from a variety of other mammals (cow, sheep, goat, pig, hamster, and mouse) hybridized with the same rat probe (Fig. 4b) indicate the presence of a gene whose sequence is cross-hybridizing with the rat oncomodulin DNA probe.

**DISCUSSION**

The oncomodulin gene is a member of a family of genes encoding various calcium-binding proteins. While the products of most of the members of this family occur naturally in normal tissues, the oncomodulin gene product has not been detected in normal embryonic and adult, human, or rodent tissues (12, 16, 17). However, it does occur extraembryonically in placentas of humans and rodents (14, 15). The study of the regulation of oncomodulin gene expression is particularly important because the gene is almost always re-expressed during neoplastic transformation of human, guinea pig, mice, and rat cells (12, 13, 16, 17).
were hybridized for 16 h with probe at described under “Experimental Procedures” (the 0.1
0.75% agarose gels and blotted onto Bionyte nylon membranes. Blots

donucleases and blot hybridized with a single-stranded probe
digests of mammalian DNAs. Lanes are as indicated.

Fig. 4. Genomic DNA digested with various restriction endonucleases and blot hybridized with a single-stranded probe
prepared from clone 9-6. Ten μg digests were electrophoresed on 0.7%
agarose gels and blotted onto Bionyte nylon membranes. Blots were
hybridized for 16 h with probe at 10° cpm/ml, washed as described under “Experimental Procedures” (the 0.1
0.1% SDS wash was omitted for blot b) and exposed to Kodak SB-3 film
for 4 days. a, normal adult rat liver. Lanes are as indicated. b, EcoRI
digests of mammalian DNAs. Lanes are as indicated.

Fig. 5. Comparison of oncomodulin, parvalbumin, and calmodulin cDNA sequences. The parvalbumin and calmodulin se-
quenences were compared previously (42) and are here related to the
5'-flanking region and first 7 codons of the oncomodulin cDNA. The numbers at the beginning and end of each sequence correspond to
cDNA positions. I indicates the oncomodulin translation initiation
codon which is aligned with the same codon in the parvalbumin
cDNA. Gaps have been inserted to maximize homology.

We have shown by in vitro translation studies that onco-
modulin mRNA is present in tumor poly(A+) RNA samples
but cannot be detected in RNA extracts of normal adult rat
liver tissues (36). This result suggests that expression of the
oncomodulin gene is regulated at the level of transcription
and that the appearance of the protein in the hepatoma is the
result of the normally silent gene being activated.

cDNA clones together containing the entire coding se-
quence of oncomodulin message were constructed from sam-
ple of RNA from rat hepatoma tissue. DNA sequencing
revealed a single open reading frame containing the infor-
mation for a peptide of 108 amino acids. The amino acid
sequence deduced from the nucleotide sequence agreed exactly
with the amino acid sequence previously determined (37) with
the exception of position 25 where the Gln residue should have
read Glu. By primer extension analyses, the 5'-noncod-
ing-region of the oncomodulin message was estimated to be
97 ± 2 nucleotides long, indicating that a sequence of approxi-
matelty 21 nucleotides at the extreme 5'-end of the message is
missing from our cDNA clone. The nucleotide sequence of the
coding region of the oncomodulin cDNA was not signifi-
cantly homologous to those of cDNAs coding for other mem-
bers of the calcium-binding protein family such as rat S-100
protein (38), rat intestinal calcium-binding protein (39),
chicken (40), or eel (41) calmodulin. On the other hand the
oncomodulin sequence was 59% homologous to that of a
recently published cDNA nucleotide sequence coding for par-
valbumin (42). This is not surprising since the two proteins,
although immunologically dissimilar, have amino acid se-
quences that are 55% identical (18).

The translation initiation codon region of a parvalbumin
cDNA has recently been compared (42) to the 3'-end of the
first calcium-binding domain of a chicken calmodulin cDNA
(40) and found to be 81% identical over 32 nucleotides. It was
suggested that this homology may be due to the remnants of a
fourth calcium-binding domain in parvalbumin (42). Fig. 5
shows the alignment of the oncomodulin and parvalbumin
(42) cDNA sequences in the region of the translation initia-
tion codons. The oncomodulin sequence is in turn compared to
the 3'-end of the first calcium-binding domain of a chicken
calmodulin cDNA (40). We have found that while the onco-
modulin and parvalbumin cDNA sequences are 52% identical,
the oncomodulin and calmodulin sequences show only 38%
identity. The oncomodulin cDNA shows far less evidence of
the remains of a fourth calcium-binding domain, indicating
perhaps that the oncomodulin gene has diverged much more
from that of calmodulin than has the parvalbumin gene.

Restriction digests of genomic DNA from normal rat liver
and hepatoma tissue hybridized to the oncomodulin probe show
only one fragment in each digest, suggesting that onco-
modulin is a single copy gene. Furthermore, identical hybrid-
ization patterns in both the normal liver and hepatoma digests
indicate that expression of oncomodulin in the tumor is not the
result of a translocation of the gene. Also, the expression of the
oncomodulin gene in the hepatoma is not the result of a
gene amplification event because the intensities of the
hybridization signals appear to be the same.

The ability of the rat probe to cross-hybridize with se-
quences in DNA of humans and other mammals, points to a
conservation of the oncomodulin sequence and allows for a
study of the gene's phylogeny. Indeed, preliminary results
show that the rat probe hybridizes with reptilian, amphibian,
and avian genomic DNA fragments.

The natural pattern of expression of the oncomodulin gene
and its re-expression in proliferating tumor cells, but not
proliferating normal cells, in adult animals and humans sug-
ests a role for the protein in early embryogenesis and in
cancer cells which is not related to proliferation. The availa-
bility of a DNA probe may help to determine this role of the
oncomodulin protein. Furthermore, the study of the onco-
modulin gene and its controlling elements may lead to a better
understanding of the links between normal embryonic develop-
ment and the tumorigenic process.

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