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cDNA Cloning, Genomic Structure, and Chromosome Mapping of the Human Epithelial Membrane Protein CL-20 Gene (EMP1), a Member of the PMP22 Family

YAN CHEN,[1,2] ALEX MEDVEDEV,[2] PETER RUZANOV, KEITH W. MARVIN,[3] AND ANTON M. JETTEN[4]

Cell Biology Section, Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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CL-20 **is a novel gene encoding a protein that is structurally related to but distinct from the peripheral myelin protein PMP22. Like PMP22, CL-20 is likely to play important roles in the regulation of cell proliferation, differentiation, and cell death. In this study, we describe the cloning and sequencing of a cDNA encoding the human homologue of CL-20 and characterize the genomic structure of this gene. The *hCL-20* gene (HGMW-approved symbol EMP1) encodes a protein of 157 amino acids that exhibits 76% identity to the rabbit CL-20 and to the rat EMP-1, which have been described recently, and 39% identity to human PMP22. CL-20 contains four hydrophobic domains, suggesting that it is an integral membrane protein. In particular the second hydrophobic domain encoded within the fourth exon is highly conserved among CL-20, EMP-1, and PMP22, suggesting a functional role for this region. CL-20 mRNA is abundant in squamous-differentiated bronchial epithelial cells; however, low levels of CL-20 mRNA can be detected in several human tissues by Northern analysis. Retinoic acid, which inhibits squamous differentiation, represses CL-20 expression in normal human bronchial epithelial cells. The genomic structure of the *hCL-20* gene was analyzed using a P1 vector containing this gene. The *hCL-20* gene contains five exons about 0.2, 0.12, 0.1, 0.14, and 2.2 kb and four introns about 15, 1.9, 0.1, and 0.7 kb. We have mapped the *hCL-20* gene to chromosome 12p12 by fluorescence *in situ* hybridization.** © 1997 Academic Press

INTRODUCTION

Squamous differentiation is a multistep process that can be observed in epithelial cells of many different tissues. Early in this terminal pathway of differentiation, cells undergo irreversible growth arrest that is accompanied by a down-regulation of several cell cycle control genes, including *RB* and *cdc2* (Jetten *et al.*, 1992; Saunders and Jetten, 1994). This is followed by the induction of differentiation-specific genes such as expression of several keratins and proteins involved in the formation of the cross-linked envelope such as transglutaminase type I and cornifins (Eckert and Rorke, 1989; Jetten *et al.*, 1992; Marvin *et al.*, 1992). Recently, we cloned a cDNA CL-20 encoding a novel protein from a cDNA library prepared from squamous-differentiated rabbit tracheal epithelial cells (Marvin *et al.*, 1995). This gene is expressed at low levels in undifferentiated tracheobronchial cells and becomes highly expressed when these cells undergo squamous differentiation. The rabbit CL-20 protein exhibits a 75% identity with the recently reported epithelial membrane protein, EMP-1 (Taylor *et al.*, 1995), which might be the rat homologue of CL-20. The rabbit CL-20 exhibits moderate (43%) homology to the human peripheral myelin protein, PMP22 protein (also known as gas3) (Manfioletti *et al.*, 1990; Welcher *et al.*, 1991; Snipes *et al.*, 1992; Suter *et al.*, 1992). CL-20, EMP-1, and PMP22 exhibit a great structural similarity, suggesting that they belong to the same gene subfamily. Point mutations in the mouse *PMP22* gene are responsible for the severe myelin deficiencies and excessive proliferation of Schwann cells that underlie the neuropathies in trembler and trembler-J mice (Suter *et al.*, 1992). Genetic alterations in the *PMP22* gene have also been implicated in several neurological disorders in humans, such as Charcot–Marie–Tooth disease and Dejerine–Sottas syndrome (Valentijn *et al.*, 1992; Matsunami *et al.*, 1992; Timmerman *et al.*, 1992; Patel *et al.*, 1992; Roa *et al.*, 1993; Patel and Lupski, 1994). Furthermore, genetic alterations in the *PMP22* gene have been linked to hereditary neuropathy with liability to pressure palsies (Chance *et al.*, 1994; Nicholson *et al.*, 1994). Transgenic rats that overexpress PMP22

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[1] Present address: Blood Research Institute, Blood Center of Southeastern Wisconsin, P.O. Box 2178, Milwaukee, WI 53233.

[2] The first two authors contributed equally to the paper.

[3] Present address: Department of Pharmacology & Clinical Pharmacology, University of Auckland, Auckland, New Zealand.

[4] To whom correspondence should be addressed. Telephone: (919) 541-2768. Fax: (919) 541-4133. E-mail: jetten@niehs.nih.gov.

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exhibit abnormalities closely resembling those observed in patients with Charcot–Marie–Tooth disease (Sereda *et al.*, 1996). PMP22 is up-regulated during growth arrest of mouse NIH 3T3 fibroblasts and has a pronounced effect on the length of the G1 phase of the cell cycle in cultured Schwann cells (Ciccarelli *et al.*, 1990; Zoidl *et al.*, 1995). Recently, it has been demonstrated that overexpression of PMP22 in NIH 3T3 cells results in apoptosis (Fabbretti *et al.*, 1995). These observations suggest that PMP22 plays an important structural role in maintaining myelin compaction in peripheral nerves and has additional functions, including regulation of cell proliferation and differentiation, in some nonneural cell types.

In this study, we have cloned and sequenced a cDNA encoding the human homologue of the epithelial membrane protein CL-20[5] and compared its amino acid sequence and structure to those of the rabbit homologue and to those of rat EMP-1 and human PMP22. We have examined its expression pattern in several human tissues and demonstrated that CL-20 is highly induced when normal human bronchial epithelial cells undergo squamous differentiation. In addition, we characterized the genomic structure of this gene and mapped it to human chromosome 12p12 using fluorescence *in situ* hybridization (FISH).

MATERIALS AND METHODS

RT-PCR. The first-strand cDNA was reverse transcribed using the first-strand cDNA synthesis kit (Clontech), with 1 μg of total RNA isolated from squamous-differentiated (grown without retinoic acid) or mucous secretory (grown in the presence of retinoic acid) human bronchial epithelial cells (obtained for Dr. Karen Guzman, NIEHS) as template. This human cDNA was used in PCR to amplify the homologous region of human CL-20. The PCR primers U1 (5′-GCATCTGTAGGTCTTTGGAG) and U7 (5′-TTTCTCAGGACCAGATAGAG) were designed after the coding sequence of rabbit CL-20 (Marvin *et al.*, 1995). The PCR amplification mixture (100 μl) contained 1× PCR buffer, 1.5 m*M* $MgCl_2$, 200 μM each of the four dNTPs, 0.5 μM U1 primer, 0.5 μM U7 primer, 10 μl diluted cDNA, and 5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). The PCR was carried out as 30 cycles of 94, 55, and 72°C for 1 min each and 1 cycle of 72°C for 7 min in a DNA thermal cycler 480 (Perkin–Elmer/Cetus). A PCR product of the expected size (370 bp) was isolated from the agarose gel, directly subcloned with the TA cloning kit (Invitrogen), and sequenced. It was then used as a probe to screen a human lung cDNA library.

cDNA library screening. A human lung cDNA library constructed in Lambda ZAP (Stratagene) was obtained from Dr. Shu Wu (NIEHS). The 370-bp RT-PCR product was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) using the Prime-it RmT Random Primer Labeling kit (Stratagene) and purified using the NucTrap probe purification column (Stratagene). About 5×10^5 plaques were screened. The plaques were lifted onto Hybond-N membranes (Amersham). DNA was denatured, neutralized, and cross-linked to the membranes using the Stratalinker 2400 (at 120,000 μJ of UV energy; Stratagene). Hybridization was carried out using the Quikhyb rapid hybridization solution (Stratagene). Twenty-nine positive clones were obtained, of which 4 containing the largest insert were purified. The cloned inserts were excised from the lambda vector *in vivo* to form the pBluescript SK(−) phagemids using ExAssist helper phage and the SOLR strain (Stratagene). All 4 inserts contained a poly(A)$^+$ tail and encoded various lengths of hCL-20. The cDNA clone hCL-20-5 containing an insert of 2.8 kb encoded virtually the whole hCL-20 mRNA and was used in further experiments.

DNA sequencing. Plasmid and phagemid DNA were purified using Wizard miniprep or midiprep kits (Promega). Manual sequencing was performed using the dideoxynucleotide chain-termination method and the Sequenase Quick-denature plasmid sequencing kit (Amersham). Automatic sequencing was carried out using a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer) and an ABI Prism 377 automatic sequencer. DNA and deduced protein sequences were analyzed by the UWGCG (Devereux *et al.*, 1984) and MacVector (IBI) sequence analysis software packages.

Isolation of P1 vector clones. P1 vector clones containing 75- to 150-kb fragments of genomic DNA encoding the human CL-20 gene were obtained by screening a P1 vector library by PCR using two specific primers (Pierce *et al.*, 1992; Genome Systems, St. Louis, MO). The forward primer 100F starting at nt 100, 5′-GTAGATGCATCAGTAGGTCTT, and the reverse primer 246R starting at nt 246, 5′-GAAGACCAGGAGGGCAATGAC, were designed from the human CL-20 coding sequence described in this paper. The following amplification cycles were employed: 30 cycles of 94, 60, and 72°C for 1 min each and 1 cycle of 72°C for 7 min. This yielded one specific PCR product of about 250 bp from human placental genomic DNA (Oncogene Science, Uniondale, NY). One of the P1 vector clones was used for further investigation. DNA from this clone was digested with *Bam*HI and/or *Hin*dIII, and fragments were analyzed by Southern analysis using the complete cDNA insert (2.8 kb) from hCL-20-5 as a probe. Fragments hybridizing to the CL-20-5 probe were subcloned into Bluescript SKII and sequenced. The size of the first intron was determined by PCR using a Long PCR kit (Boehringer Mannheim).

Fluorescence in situ hybridization. The regional chromosomal localization was determined by FISH using fragments of genomic DNA containing the human *CL-20* gene cloned into P1 vectors as a probe (Stokke *et al.*, 1995). The FISH was carried out by Genome Systems. Two different methods were employed. In the first method, DNA was labeled with digoxigenin–dUTP by nick-translation. Labeled probe was combined with sheared DNA and hybridized to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes from a male donor. Specific hybridization signals were detected by incubating the hybridized slides with fluoresceinated anti-digoxigenin antibodies followed by counterstaining with 4′,6 diamidino-2-phenylindole. In the second method, the labeled P1 vector DNA was cohybridized with a biotin-labeled probe specific for the centromere of chromosome 12. The latter results in the specific labeling of the centromeric region of chromosome 12 after incubation with Texas red-labeled avidin. A total of 80 metaphase cells were analyzed, of which 65 exhibited specific labeling.

Northern blot analysis. Human multiple tissue Northern blots were purchased from Clontech. A blot containing total RNA isolated from human bronchial epithelial cells cultured with and without retinoic acid (100 n*M*) was obtained from Dr. Karen Guzman (NIEHS). The blots were hybridized using ^{32}P-labeled probes for CL-20, transglutaminase type I (Floyd and Jetten, 1989), β-actin (Clontech), and glyceraldehyde-3-phosphate dehydrogenase (pGAD28; Dugaiczyk *et al.*, 1983) under the same conditions as described above for cDNA library screening.

RESULTS AND DISCUSSION

Cloning of human CL-20 cDNA. With primers U1 and U7 (Materials and Methods) designed from the coding sequence of the rabbit CL-20 (Marvin *et al.*, 1995), we succeeded in amplifying a specific RT-PCR product of 370 bp from total RNA isolated from human

[5] The HGMW-approved symbol for the gene described in this paper is EMP1.

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AAACACGGTGAAAAGAAACCGCCCATTACACACCCCAGTACACCAGCAGAGGAAACTTAT 60
AACCTCGGGAGGCAGGTCCTTCCCCTCAGTGCGGTCACATACTTCCAGAAGAGCGGACCA 120
GGGCTGCTGCCAGCACCTGCCACTCAGAGCGCCTCTGTCGCTGGGACCCTTCAGAACTCT 180
CTTTGCTCACAAGTTACCAAAAAAAAAAGAGCCAACATGTTGGTATTGCTGGCTGGTATC 240
                                    M  L  V  L  L  A  G  I   8

TTTGTGGTCCACATCGCTACTGTTATTATGCTATTTGTTAGCACCATTGCCAATGTCTGG 300
F  V  V  H  I  A  T  V  I  M  L  F  V  S  T  I  A  N  V  W  28

TTGGTTTCCAATACGGTAGATGCATCAGTAGGTCTTTGGAAAAACTGTACCAACATTAGC 360
L  V  S  N  T  V  D  A  S  V  G  L  W  K  N  C  T  N  I  S  48

TGCAGTGACAGCCTGTCATATGCCAGTGAAGATGCCCTCAAGACAGTGCAGGCCTTCATG 420
C  S  D  S  L  S  Y  A  S  E  D  A  L  K  T  V  Q  A  F  M  68

ATTCTCTCTATCATCTTCTGTGTCATTGCCCTCCTGGTCTTCGTGTTCCAGCTCTTCACC 480
I  L  S  I  I  F  C  V  I  A  L  L  V  F  V  F  Q  L  F  T  88

ATGGAGAAGGGAAACCGGTTCTTCCTCTCAGGGGCCACCACACTGGTGTGCTGGCTGTGC 540
M  E  K  G  N  R  F  F  L  S  G  A  T  T  L  V  C  W  L  C  108

ATTCTTGTGGGGGTGTCCATCTACACTAGTCATTATGCGAATCGTGATGGAACGCAGTAT 600
I  L  V  G  V  S  I  Y  T  S  H  Y  A  N  R  D  G  T  Q  Y  128

CACCACGGCTATTCCTACATCCTGGGCTGGATCTGCTTCTGCTTCAGCTTCATCATCGGC 660
H  H  G  Y  S  Y  I  L  G  W  I  C  F  C  F  S  F  I  I  G  148

GTTCTCTATCTGGTCCTGAGAAAGAAATTAAGGCCGGACGAGTTCATGGGGATCTGGGGGG 720
V  L  Y  L  V  L  R  K  K  *                                  157

TGGGGAGGAGGAAGCCGTTGAATCTGGGAGGGAAGTGGAGGTTGCTGTACAGGAAAAACC 780
GAGATAGGGGAGGGGGGAGGGGGAAGCAAAGGGGGGAGGTCAAATCCCAAACCATTACTG 840
AGGGGATTCTCTACTGCCAAGCCCCTGCCCTGGGGAGAAAGTAGTTGGCTAGTACTTTGA 900
TGCTCCCTTGATGGGGTCCAGAGAGCCTCCCTGCAGCCACCAGACTTGGCCTCCAGCTGT 960
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GGTCATGCACTGAGGTCCACAGACCTACTGCACTGAGTTAAAATAGCGGTACAAGTTCTG 1080
GCAAGAGCAGATACTGTCTTTGTGCTGAATACGCTAAGCCTGGAAGCCATCCTGCCCTTC 1140
TGACCCAAAGCAAAACATCACATTCCAGTCTGAAGTGCCTACTGGGGGGCTTTGGCCTGT 1200
GAGCCATTGTCCCTCTTTGGAACAGATATTTAGCTCTGTGGAATTCAGTGACAAAATGGG 1260
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ATATAATTGTGAAGTATTAAGCCTACCGTATTTCAGCCATGATAAGAACAGAGTGCCTGC 1920
ATTCCCAGGAAAATACGAAAATCCCATGAGATAAATAAAAATATAGGTGATGGGCAGATC 1980
TTTTCTTTAAAATAAAAAAGCAAAAACTCTTGTGGTACCTAGTCAGATGGTAGACGAGCT 2040
GTCTGCTGCCGCAGGAGCACCTCTATACAGGACTTAGAAGTAGTATGTTATTCCTGGTTA 2100
AGCAGGCATTGCTTTGCCCTGGAGCAGCTATTTTAAGCCATCTCAGATTCTGTCTAAAGG 2160
GGTTTTTTGGGAAGACGTTTTCTTTATCGCCCTGAGAAGATCTACCCCAGGGAGAATCTG 2220
AGACATCTTGCCTACTTTTCTTTATTAGCTTTCTCCTCATCCATTTCTTTTATACCTTTC 2280
CTTTTTGGGGAGTTGTTATGCCATGATTTTTGGTATTTATGTAAAAGGATTATTACTAAT 2340
TCTATTTCTCTATGTTTATTCTAGTTAAGGAAATGTTGAGGGCAAGCCACCAAATTACCT 2400
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CATGTATTCCCTTATCTTTTACTTTTTTTCTGTGACATTTATGTCTCATGTAATTTGCAT 2640
TACTCTGGTGGATTGTTCTAGTACTGTATTGGGCTTCTTCGTTAATAGATTATTTCATAT 2700
ACTATAATTGTAAATATTTTGATACAAATGTTTATAACTCTAGGGATATAAAAACAGATT 2760
CTGATTCCCA(n)
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FIG. 1. Nucleotide and deduced amino acid sequence of human CL-20. The amino acid sequence is noted below the nucleotide sequence. The translation start site (nucleotide 217), the stop codon (nucleotide 688), and the polyadenylation signal (nucleotide 2747) are shown in boldface and are underlined. Three AUUUA instability motifs in the 3′-UTR are also indicated in boldface. Nucleotides and amino acids are numbered on the right side of the sequence.

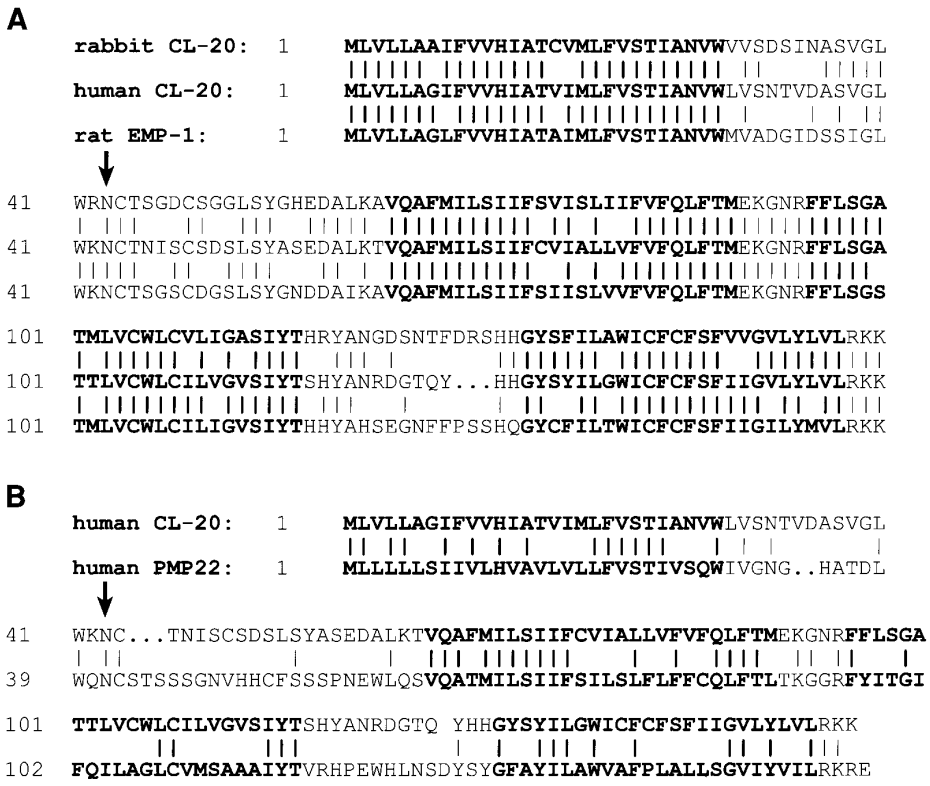


FIG. 2. Comparison between the amino acid sequence of hCL-20 and several family members. Vertical lines indicate identical amino acids. The conserved, potential N-glycosylation site is indicated by an arrow, and the four conserved hydrophobic domains are indicated by boldface letters. (**A**) Alignment of the amino acid sequences of human CL-20, rabbit CL-20, and rat EMP-1. (**B**) Alignment of the amino acid sequences of human CL-20 and human PMP22.

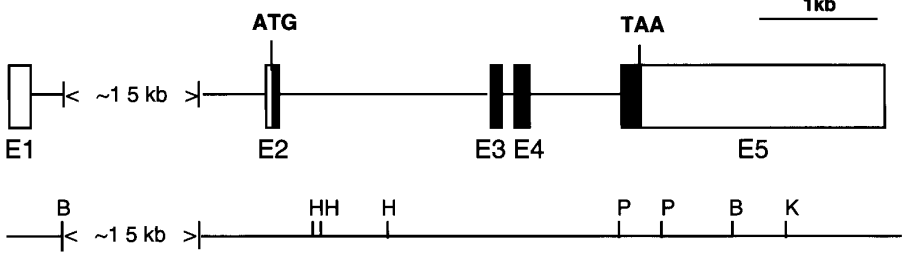
bronchial epithelial cells. Sequence analysis showed that this PCR fragment was highly similar to rabbit CL-20 and likely part of the human homologue of this gene.

To obtain the full-length cDNA of human CL-20, we screened a human lung cDNA library using this fragment as a probe. From 5×10^5 plaques screened, 29 putative positive clones were obtained in the initial screening, of which 4 were purified. The cloned inserts were excised and cloned into the pBluescript SK(−) phagemid for sequencing analysis. The sequences of the 4 cDNA clones were analyzed and found to be identical. All four clones contained a poly(A)$^+$ tail, 3′-untranslated region (3′-UTR) and full coding region but varied in the length of their 5′-UTR. The cDNA clone hCL-20-5, containing the longest insert of 2.8 kb, encoded virtually the whole hCL-20 mRNA. A putative translation start site was found 217 nucleotides from the 5′-end of the cDNA. The open reading frame terminated with a stop codon 472 nucleotides downstream from the initiation codon, indicating that it encodes a protein of 157 amino acids (Fig. 1). hCL-20 contains a large 3′-untranslated region of about 2 kb that contains three AUUUA instability motifs and a putative TATAAA polyadenylation signal.

Protein sequence comparison of CL-20 with PMP22 and EMP-1. As shown in Fig. 2A, the deduced human CL-20 amino acid sequence exhibits a 76% identity with the rabbit CL-20 protein (Marvin *et al.*, 1995) and the rat EMP-1 protein (Taylor *et al.*, 1995), which might constitute the rat homologue of CL-20. The amino acid sequence identity between rabbit CL-20 and rat EMP-1 is also 76%. Computer analysis identified two potential N-glycosylation sites at amino acids N_{43} and N_{46} of human CL-20, the first one being conserved in rabbit CL-20 and rat EMP-1. In addition, four hydrophobic regions spanning amino acids 1–28, 64–89, 95–117, and 131–154, which can also be found in rabbit CL-20 and rat EMP-1, were identified in human CL-20 (bold amino acids in Fig. 2A). In particular the four hydrophobic domains are highly conserved, while the hydrophilic regions show a high degree of diversity.

As shown in Fig. 2B, human CL-20 exhibits a 39% identity with the human PMP22 protein, with the conserved amino acids predominantly located within the four hydrophobic domains. The highest identity (91%) is found in an 11-amino-acid region (VQATMILSIIF) within the second hydrophobic domain. The high homology of this region between these proteins supports the concept that these amino acids constitute a functionally important domain. Recently, a structural model of PMP22 has been proposed in which the four hydrophobic domains span the membrane either as a β-sheet or as an α-helix (Patel and Lupski, 1994).

A



B

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AAACACGGTGAAAAGAAACCGCCCATTACACACCCCAGTACACCAGCAGAGGAAACTTATAACCTCGGGAGGCAGGTCCTTCCCCTCAGTGCGGTCACATACTTCCAGAAGAGCGGACCA 120 Exon 1
GGGCTGCTGCCAGCACCTGCCACTCAGAGCGCCTCTGTCGCTGGGACCCTTCAGgtaggacagctcccaacgctgtggggactctcagcaaaacttctccttcctttccacggctctgct 240
tcttctgacctcatcttagtttt                                  < ~15kb >                                                    Intron 1
tgagcaatttgatgaaagcatggacattttccttagtggaaaatggacatttatatgacattgtttcagagacctggaaattataaaggcctactttctttgaaaatcaaagtcctattt 120
ttaacctctaaaacacaaacaaaataaaatacgaacagttatcataaaatgttcttgttccttttttgaaatagaatgtattagagaaatgagtatgtaaatacccaagaagaaaccaaa 240
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AAAAAAAAAAGAGCCAACATGTTGGTATTGCTGGCTGGTATCTTTGTGGTCCACATCGCTACTGTTATTATGCTATTTGTTAGCACCATTGCCAATgtgagtaatgttcttttgttcatt 600 Exon 2
                  M  L  V  L  L  A  G  I  F  V  V  H  I  A  T  V  I  M  L  F  V  S  T  I  A  N                          26
cattcattgagaaatcattcgaatatttacatcaagtgcacaaaagaagtttaagccacagcccttgcccttcatgaacttacagctcagttgtgggaaaacaaaataaacacacgcttg 720
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tgttattttattttaaatcagagggccagctttcaattaacatttctttccttctggtttcagGTCTGGTTGGTTTCCAATACGGTAGATGCATCAGTAGGTCTTTGGAAAAACTGTA 2520 Exon 3
                                                                                  V  W  L  V  S  N  T  V  D  A  S  V  G  L  W  K  N  C  T
CCAACATTAGCTGCAGTGACAGCCTGTCATATGCCAGTGAAGgtatatataaagatattgacccagtgctgacaataggtgtggtcagggaatgttcagcccctggagtgacctcatgcc 2640 Intron 3
  N  I  S  C  S  D  S  L  S  Y  A  S  E  D
cttgttctccttgtccgtgtgtctacagATGCCCTCAAGACAGTGCAGGCCTTCATGATTCTCTCTATCATCTTCTGTGTCATTGCCCTCCTGGTCTTCGTGTTCCAGCTCTTCACCATG 2760 Exon 4
                        A  L  K  T  V  Q  A  F  M  I  L  S  I  I  F  C  V  I  A  L  L  V  F  V  F  Q  L  F  T  M
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E  K  G  N  R  F  F  L  S  G  A  T  T  L  V  C  W
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gacaggacagctgggtggtaggcatgaggttctgtgttttacatcacagcgtggccacacaaaccagtgcagacatgagagcccagacacctctaatttatcaacatacgtcgagcttg 3360
gcccaggggacatccatcagaactcctgtttagggacaaacaagcagaattcattttcctattgctaatgagggaaataggatcttcttaaaattagtaaaactttttctcgactttaat 3480
gtaagaaaacacaccgacaaatctccttttccccctgcagGGCTGTGCATTCTTGTGGGGGTGTCCATCTACACTAGTCATTATGCGAATCGTGATGGAACGCAGTATCACCACGGCTATT 3600
                                        L  C  I  L  V  G  V  S  I  Y  T  S  H  Y  A  N  R  D  G  T  Q  Y  H  H  G  Y  S
CCTACATCCTGGGCTGGATCTGCTTCTGCTTCAGCTTCATCATCGGCGTTCTCTATCTGGTCCTGAGAAAGAAATAAGGCCGGACGAGTTCATGGGGATCTGGGGGGTGGGGAGGAGGAA 3720
  Y  I  L  G  W  I  C  F  C  F  S  F  I  I  G  V  L  Y  L  V  L  R  K  K
GCCGTTGAATCTGGGAGGGAAGTGGAGGTTGCTGTACAGGAAAAACCGAGATAGGGGAGGGGGGAGGGGGAAGCAAAGGGGGGAGGTCAAATCCCAAACCATTACTGAGGGGATTCTCTA 3840
CTGCCAAGCCCCTGCCCTGGGGAGAAAGTAGTTGGCTAGTACTTTGATGCTCCCTTGATGGGGTCCAGAGAGCCTCCCTGCAGCCACCAGACTTGGCCTCCAGCTGTTCTTAGTGACACA 3960 Exon 5
CACTGTCTGGGGCCCCATCAGCTGCCACAACACCAG.....(3'-UTR as in Fig. 1).....GCAAAAAACATACTTACATTTCAGACATATCCAAAG 5520
GGAATACTCACATTTTGTTAAGAAGTTGAACTATGACTGGAGTAAACCATGTATTCCCTTATCTTTTACTTTTTTTCTGTGACATTTATGTCTCATGTAATTTGCATTACTCTGGTGGAT 5640
TGTTCTAGTACTGTATTGGGCTTCTTCGTTAATAGATTATTTCATATACTATAATTGTAAATATTTTGATACAAATGTTTATAACTCTAGGGATATAAAAACAGATTCTGATTCCC     5756
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Based on its structural similarity with PMP22, CL-20 probably also functions as a membrane protein. Both rabbit CL-20 and human PMP22 are able to bind the monoclonal antibody NHK-1, which recognizes epitopes composed of sulfated carbohydrates, indicating that CL-20 and PMP22 are glycosylated (Hammer *et al.*, 1993; Snipes *et al.*, 1993; Jetten, unpublished observations). This glycosylation most likely occurs at the highly conserved, potential glycosylation site N_{43}. Although the function of this epitope in CL-20 and PMP22 has yet to be identified, the HNK-1 epitope has been shown to play a role in cell–cell recognition processes in other proteins (Schachner and Martini, 1995).

Most mutations in PMP22 associated with hereditary motor and sensory neuropathies are located within the membrane-spanning domains (Patel and Lupski, 1994). Several mutations have been identified within the highly conserved 11-amino-acid region (VQAFMILSIIF) in the second hydrophobic domain and involve amino acids that are identical between PMP22 and CL-20. This provides additional support for the functional importance of this region. In addition, one may speculate that putative mutations in the same region of CL-20 could be involved as well in certain disease processes.

Structure of the hCL-20 gene. P1 vector clones containing 75- to 100-kb fragments of genomic DNA that include the *CL-20* gene were isolated by screening a P1 vector library by PCR using two specific primers, 100F and 246R, that under appropriate conditions yielded a PCR product of about 250 bp with human genomic DNA as template. P1 vector DNA was cut with *Bam*HI and subjected to Southern analysis using ^{32}P-hCL-20-5 as a probe. The fragments hybridizing to the complete cDNA fragment of hCL-20-5 were subcloned into Bluescript and sequenced. The *hCL-20* gene contained five exons and four introns (Fig. 3). The nucleotide sequence of the exons was identical to that obtained for the hCL-20-5 cDNA. The first intron in the *hCL-20* gene was approximately 15 kb and located within the 5′-UTR between G_{174} and A_{175} of the hCL-20-5 cDNA. The other introns were located in the coding region. The second intron of about 1.9 kb was located between T_{294} and G_{295}, the third intron of 102 bp between nucleotides G_{391} and A_{392}, and the fourth intron of 710 bp between nucleotides T_{532} and G_{533}. A summary of the exon/intron junctions is presented in Table 1. The sequences of these junctions are consistent with the consensus ($A_{62}G_{77}/g_{100}t_{100}a_{60}a_{74}g_{88}$ for the 5′ donor and $y_{87}ny_{97}a_{100}g_{100}/G_{55}$ for the 3′ acceptor side) for known splice sites within eukaryotic genes (Mount, 1982). It is interesting to note that the exon/intron junctions interrupt the coding sequence of human *CL-20* at the same locations as reported for *PMP22* (Suter *et al.*, 1994), corroborating that these two genes belong to the same family and are likely derived from duplications of a common ancestral gene.

TABLE 1

DNA Sequences of the Exon/Intron Junctions of Human *CL-20*

5′ exon/intron junction	Intron size (kb)	3′-exon/intron junction
$CTTCAG_{174}$gtagga	~15	tttcagA_{175}ACTCT
$GCCAAT_{294}$gtgagt	1.889	tttcagG_{295}TCTGG
$GTGAAG_{391}$gtatat	0.102	ctacagA_{392}TGCCC
$TGTGCT_{532}$gtgagt	0.71	tgcagG_{533}GCTGT

Note. Exon sequences are shown in uppercase letters and intron sequences in lowercase letters. The nucleotide numbers indicate the 5′- and 3′-positions in the human CL-20-5 cDNA (Fig. 1) that flank each intron.

Chromosomal localization of human CL-20. The regional chromosomal localization was determined by FISH using fragments of genomic DNA containing the human *CL-20* gene cloned into P1 vectors as a probe (Stokke *et al.*, 1995). DNA of a positive P1 clone was labeled with digoxigenin and hybridized to metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes from a male donor. Results of this analysis indicated that *CL-20* is localized on human chromosome 12. This was confirmed by the colocalization of a biotin-labeled probe specific for the centromere of chromosome 12. The regional assignment of the CL-20 probe was determined by the analysis of the fractional chromosome length; 10 specifically hybridized chromosomes were measured. These calculations indicated that the *CL-20* gene mapped to a position that is 56% of the distance from the centromere to the telomere of chromosome arm 12p, an area that corresponds to 12p12. The result of the *in situ* hybridization is shown in Fig. 4A. An idiogram indicating the chromosomal localization of human *CL-20* is presented in Fig. 4B. The mapping of *hCL-20* to chromosome 12 by FISH is in agreement with our previous findings using a panel of hybrid human/hamster cell lines (Marvin *et al.*, 1995). The localization of *hCL-20* is distinct from that of *hPMP22*, which has recently been mapped to chromosome 17p11.2–p12 (Martinotti *et al.*, 1992; Matsunami *et al.*, 1992; Roa *et al.*, 1993). Mutations and duplications in the latter gene have been found in asso-

FIG. 3. Genomic structure of the human *CL-20* gene. (**A**) Schematic presentation of the genomic structure of the human *CL-20* gene. The start codon (ATG) and stop codon (TAA) are indicated. Boxes indicate exons; black boxes indicate coding region. B, H, S, and K represent *Bam*HI, *Hind*III, *Pst*I, and *Kpn*I, respectively, (**B**) Sequenced region of the human *CL*-20 gene. Uppercase letters indicate exon sequences and lowercase letters intron sequences. The coding regions are marked by single-letter amino acid code. The sequence of the complete 3′-UTR was obtained from genomic DNA and found to be identical to that obtained from genomic DNA and found to be identical to that obtained from the cDNA clone hCL-20-5 shown in Fig. 1. The sequence has been submitted to GenBank under Accession No. U77085.

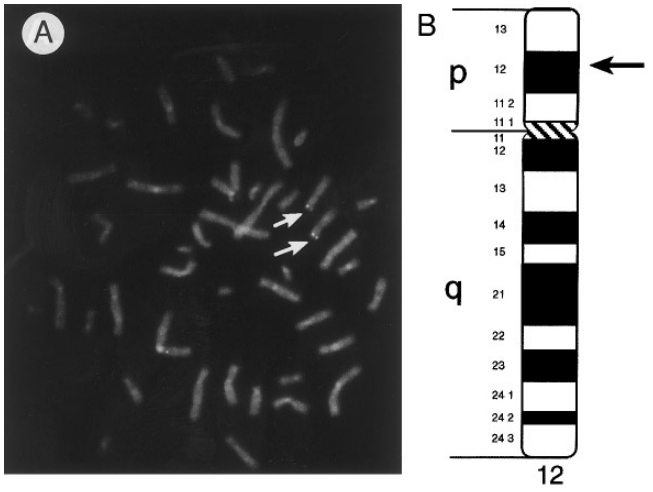

FIG. 4. Regional mapping of the *hCL-20* gene by fluorescence *in situ* hybridization to human chromosome 12p12. (**A**) The location of the *hCL-20* gene was identified using a digoxigenin–dUTP-labeled genomic fragment containing the *hCL-20* gene. Colocalization on chromosome 12 was determined by cohybridization with a biotinylated probe specific for the centromere of chromosome 12. Only the staining for *hCL-20* is shown. (**B**) The idiogram indicates that *hCL-20* maps to band 12p12. The arrow indicates the interval within which the hybridization signal was detected on a sample of 80 chromosomes. No specific signal was detected on other chromosomes.

ciation with several neuropathies (Valentijn *et al.*, 1992; Matsunami *et al.*, 1992; Roa *et al.*, 1993; Patel and Lupski, 1994; Chance *et al.*, 1994; Nicholson *et al.*, 1994). Various genetic alterations involving the 12p12 locus have been identified in breast fibrosarcomas (Calabrese *et al.*, 1991), lymphoblastic leukemia (Cave *et al.*, 1995), and cardiac myxoma (Dijkhuizen *et al.*, 1995). Future studies must determine what role the *CL-20* gene plays in disease.

Tissue distributions of human CL-20 mRNA. To examine the pattern of CL-20 expression in different human tissues, we analyzed the levels of CL-20 mRNA by Northern blot analysis. As shown in Fig. 5, the 2.8-kb CL-20 transcript can be detected in many of the tissues examined, including heart, placenta, lung, skeletal muscle, kidney, spleen, thymus, prostate, testis, ovary, small intestine, and colon; but not in brain, liver, pancreas, or peripheral blood leukocytes. A β-actin probe was used to compare the relative level of RNA loaded. Several differences in the expression pattern between CL-20 and EMP-1 are noticeable. In particular the expression of CL-20 in the colon appears less than that reported for EMP-1 (Taylor *et al.*, 1995). Future studies must elucidate whether CL-20 and EMP-1 are homologues of the same gene or represent two different members of this gene family.

The expression of CL-20 is differentially regulated by retinoids. Previous studies have shown that the induction of rabbit CL-20 mRNA expression is closely correlated with squamous differentiation and that retinoids, which inhibit squamous differentiation, also repress the induction of rabbit CL-20 (Marvin *et al.*, 1995). To examine the regulatory effect of retinoids on CL-20 expression in human cells, we analyzed the mRNA level in human bronchial epithelial (HBE) cells cultured in the presence or absence of retinoic acid by Northern blot analysis. In the absence of retinoic acid, HBE cells undergo squamous differentiation and, as shown in Fig. 6, CL-20 was highly expressed, as indicated by the strong band representing the 2.8-kb CL-20 mRNA (lane 2). However, squamous differentiation is inhibited in the presence of retinoic acid and instead, HBE cells undergo differentiation into mucous cells (Gray *et al.*, 1996). Under this condition the expression of CL-20 was greatly suppressed, as indicated by the dramatic decrease in the hybridization signal of the 2.8-kb CL-20 transcript (Fig. 6, lane 2). The levels of CL-20 mRNA correlate with those of transglutaminase type I mRNA (Floyd and Jetten, 1989), which is not expressed in retinoic acid-treated HBE cells but is

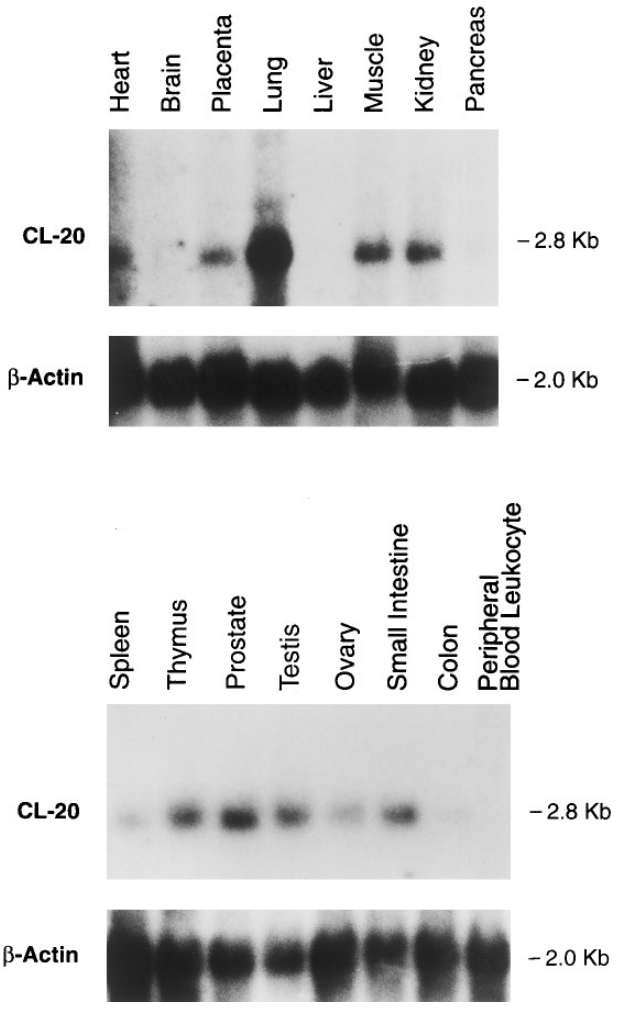

FIG. 5. Tissue-specific expression of human CL-20 mRNA. Poly(A)$^+$ RNA from different human tissues was examined by Northern blot analysis with ^{32}P-labeled CL-20 and β-actin probes as described under Materials and Methods. The 2.8-kb CL-20 and 2.0-kb β-actin transcript are indicated on the right.

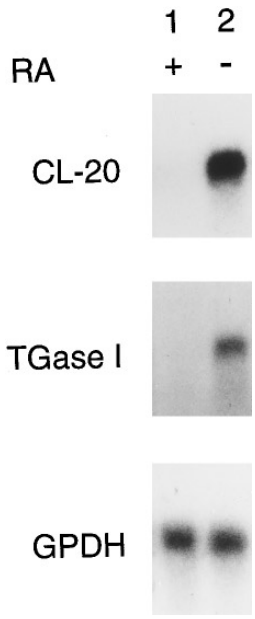

FIG. 6. Differential expression of human CL-20 in squamous-differentiated and mucous-secretory HBE cells. Total RNA from retinoic acid-treated (100 n*M*) and untreated HBE cells was examined by Northern blot analysis as described under Materials and Methods using ^{32}P-labeled probes for hCL-20, transglutaminase Type I (TGase I), and glyceraldehyde-3-phosphate dehydrogenase (GPDH). The 2.8-kb CL-20 mRNA, 2.8-kb transglutaminase type I, and the 1.3-kb GPDH mRNA are indicated.

highly expressed in squamous cells (Fig. 6). Taking into account differences in exposure time and the fact that total RNA was used instead of poly(A)$^+$ RNA, the expression of CL-20 was estimated to be at least 50 times higher in squamous-differentiating HBE cells than in any of the tissues used in Fig. 5. These results are in agreement with previous observations showing an association between high expression of CL-20 and squamous differentiation (Marvin *et al.*, 1995).

The functional roles of CL-20 and PMP22 have not been precisely established. PMP22 has been shown to be expressed in neural as well as nonneural tissues during embryonic development and in the adult (Manfioletti *et al.*, 1990; Welcher *et al.*, 1991; Baechner *et al.*, 1995). These observations support the concept that PMP22 serves, in addition to its important structural role in maintaining myelin compaction in peripheral nerves, other functions including the regulation of cellular differentiation and proliferation. The dual role of PMP22 is supported by findings demonstrating that the regulation of this gene is controlled by two promoters (Suter *et al.*, 1994). Recently, it was shown that overexpression of PMP22 induces apoptosis in NIH 3T3 fibroblasts (Fabbretti *et al.*, 1995). In addition, PMP22 is induced when 3T3 fibroblasts undergo growth arrest (Ciccarelli *et al.*, 1990), and in Schwann cells it delays the transition from the G1- to the S-phase in the cell cycle (Zoidl *et al.*, 1995). These observations suggest a role for PMP22 in the control of cell growth. It is interesting to note that the expression of CL-20 in tracheobronchial cells is induced at a time when these cells undergo terminal differentiation and become irreversibly growth-arrested (Marvin *et al.*, 1995). Whether the induction of CL-20 in these cells is in any way directly related to the control of irreversible growth arrest has yet to be determined. Experiments to determine the effect of the overexpression of CL-20 on growth and differentiation are in progress.

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Note added in proof. During review of this paper Ruegg, C. L., *et al.* (*J. Immunol.* **157:** 72–80, 1996) reported the cDNA sequence of a novel gene B4B which is identical to the human homologue of EMP1/CL-20.

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