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Cloning and molecular characterization of a novel gene strongly induced by the adenovirus E1A gene in rat thyroid cells

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Expression of the adenovirus E1A gene in the rat thyroid differentiated cell line PC Cl 3 induces thyrotropin-independent cell growth and impairs differentiation. However, the malignant phenotype is achieved only when the PC E1A cells are infected with other murine retroviruses carrying the v-*abl*, v-*raf* or polyoma middle-T genes. To determine through which genes E1A affects thyroid cells, we differentially screened PC Cl 3 and PC E1A cells. Here we report a new gene, named CL2, that is upregulated in PC E1A cells. The CL2 transcript is 4.4 kb long and encodes a 949 amino-acid protein. Conceptual translation of the open reading frame showed one product with a signal peptide, multiple nuclear localization signals and three newly described domains. Furthermore, *in vivo*, this protein was located juxtanuclear, which is suggestive of Golgian localization, and also in cytoplasm and nucleus/nucleolus. Finally, CL2 gene expression was drastically downregulated in human thyroid neoplastic cell lines and tissues.
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Introduction

The adenovirus E1A gene encodes nuclear proteins that are essential to establish a productive viral infection in human cells. Differential splicing of the E1A transcript produces a number of E1A mRNA, with a predominance of species 13S and 12S. The 13S species encodes a protein of 289 amino acids (289R protein), while the 12S mRNA produces a protein of 243 amino acids (243R). Two regions conserved among adenovirus serotypes (conserved regions 1 and 2) are common to 243R and 289R, while a third conserved region is unique to the 289R protein. In cultured cells, depending on the cell system, both E1A proteins participate in transcriptional activation and transcriptional repression, and can stimulate cells to enter into S phase by binding to negative regulators of cell growth, including the retinoblastoma protein (Rb) and the transcriptional coactivator p300 (Gallimore and Turnell, 2001).

The products of the E1A gene can also immortalize primary cells (Paraskeva and Gallimore, 1980; Cone *et al*., 1988; Lynch and Trainer, 1989) and can cooperate with the adenovirus E1B gene or other oncogenes to promote oncogenic transformation of primary rodent cells (Ruley, 1983). Interestingly, it appears that the same E1A activities required for oncogenic transformation also stimulate programmed cell death (Whyte *et al*., 1988, 1989; Chinnadurai, 1992; White, 1995, 2001). Induction of apoptosis by E1A may explain why the gene must be coexpressed with an apoptosis inhibitor for transformation to occur. Introduction of the adenovirus 5 E1A gene (Stow, 1982) into the differentiated rat thyroid cell line PC Cl 3 blocks differentiated functions (thyroglobulin gene expression and iodide uptake) and renders cell growth partially independent of thyrotropin (TSH). However, the cells remain unable to grow in semisolid medium and to induce tumors when injected into athymic mice unless they are transfected with such other oncogenes as v-*raf*, v-*src* or polyoma middle-T antigen (Berlingieri *et al*., 1993).

The aim of this study was to determine through which genes E1A exerts its effects on thyroid cells. To this purpose, we performed a differential screening between PC Cl 3 and PC E1A cells. Here we describe the identification of a novel gene, named CL2, that was expressed at very high levels in the PC E1A cells, but almost undetectable in normal PC Cl 3 cells. The CL2 gene open reading frame (ORF) encodes a 949 amino-acid protein containing three novel domains; it is also

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characterized by a signal peptide and multiple nuclear localization signals (NLS). Thanks to the latter two features, the protein can be secreted and shuttled into the nucleus. The CL2 gene may also play a role in human thyroid carcinogenesis. In fact, expression of the CL2 transcript was significantly decreased in several human thyroid neoplastic cell lines and tissues.

Results

Isolation of the CL2 gene

To isolate genes whose expression was induced by the adenovirus 5 E1A gene in PC Cl 3 cells, we performed a differential screening of a PC E1A cDNA library. Several phages hybridizing to the cDNA obtained from the PC E1A cell line and not to cDNA from normal PC Cl 3 cells were isolated. The inserts were purified and hybridized by Northern blot to RNA extracted from PC Cl 3 and PC E1A cells. The Northern blot data confirmed induction of these cDNA by the E1A oncogene (data not shown). Among these cDNA clones we investigated one whose expression was not detected in the untransfected PC Cl 3 cells, whereas it was abundantly expressed in PC E1A cells. We named this cDNA CL2, as it was the second one to be isolated in our screening. We observed a very intense signal corresponding to CL2 mRNA even after few hours of exposure (Figure 1, Panel a). Three other PC E1A cell clones plus the pooled population were also analysed for CL2 gene expression: they all showed high expression levels of the gene (data not shown). The CL2 mRNA was also expressed at comparable levels in PC HE4 cells (Figure 1, Panel a), which are PC Cl 3 cells transfected with another variant of the E1A gene deriving from adenovirus 2 (Rossini, 1983). Furthermore, there was a clear correlation between the E1A and CL2 levels in recently obtained PC E1A cell clones stably expressing E1A 1 week after the end of the selection period (Figure 1, Panel b). These results indicate that CL2 expression in PC Cl 3 cells is induced by the E1A gene and is not a clonal event.

The insert size of the CL2 clone was 1.8 kb, whereas the size of CL2 mRNA was estimated to be about 4.0 kb. Therefore, to isolate a full-length cDNA, we used the 1.8 kb CL2 insert as a probe to screen a rat brain cDNA library. Two clones, named λCL2-22 (3221 bp) and λCL2-24 (3492 bp), were obtained. They did not possess a typical polyadenylation signal. Northern blot hybridization experiments demonstrated that both clones detected a $\sim$4.0 kb band in PC E1A cells, but not in normal PC Cl 3 cells (data not shown). After matching their sequences, we obtained a 3540 bp cDNA.

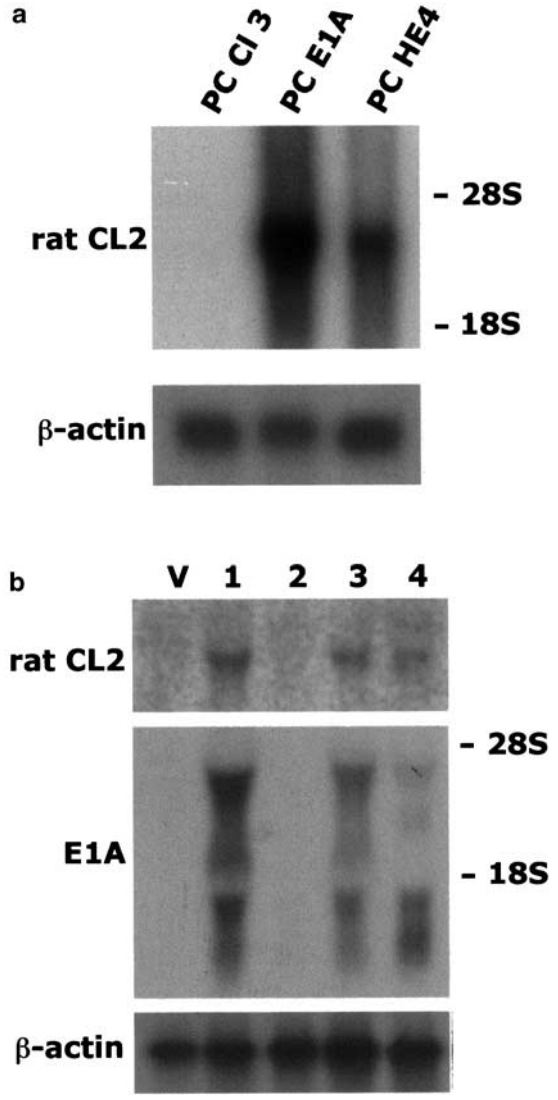


Figure 1 Analysis of the expression of the CL2 gene in PC Cl 3, PC E1A and PC HE4 cells. Panel **(a)** In all, 10 μg of total RNA for each cell line was size-fractionated on a 1.2% denaturing formaldehyde agarose gel, blotted onto Nylon filters hybond-N and probed with a 1.1 kb *EcoR*I/*Hind*III fragment from the pGem3ZCL2 vector, β-actin was used as control for uniform RNA loading. Panel **(b)** In all, 10 μg of total RNA was extracted from four cell clones (indicated by the numbers 1–4) derived from PC Cl 3 cells transfected one month before with a construct encoding the E1A 13S gene or from one clone transfected with the control vector (V). The RNAs were size-fractionated on a 1.2% denaturing formaldehyde agarose gel, blotted onto Nylon filters hybond-N and probed with a fragment from the pGem3ZCL2 vector (upper panel) or from the pSV2neo13Swt vector (middle panel). β-actin was used as control for uniform RNA loading (lower panel)

Sequence analysis of the CL2 gene

Analysis of the CL2 cDNA revealed a single long ORF. The presence of four in-frame stop codons, located upstream from the assigned translation start site, and two in-frame stop codons at the 3′ end suggest that the cDNA contains the entire coding region of the gene. This ORF has the potential to code for a 949 amino-acid protein (Figure 2) having a predicted molecular mass of 107.7 kDa. *In vitro* transcription–translation substantiated this hypothesis (Figure 3, Panel a). This highly basic protein (pI 9.6) has multiple potential nuclear localization signals starting at amino acids 494 (PKKK),

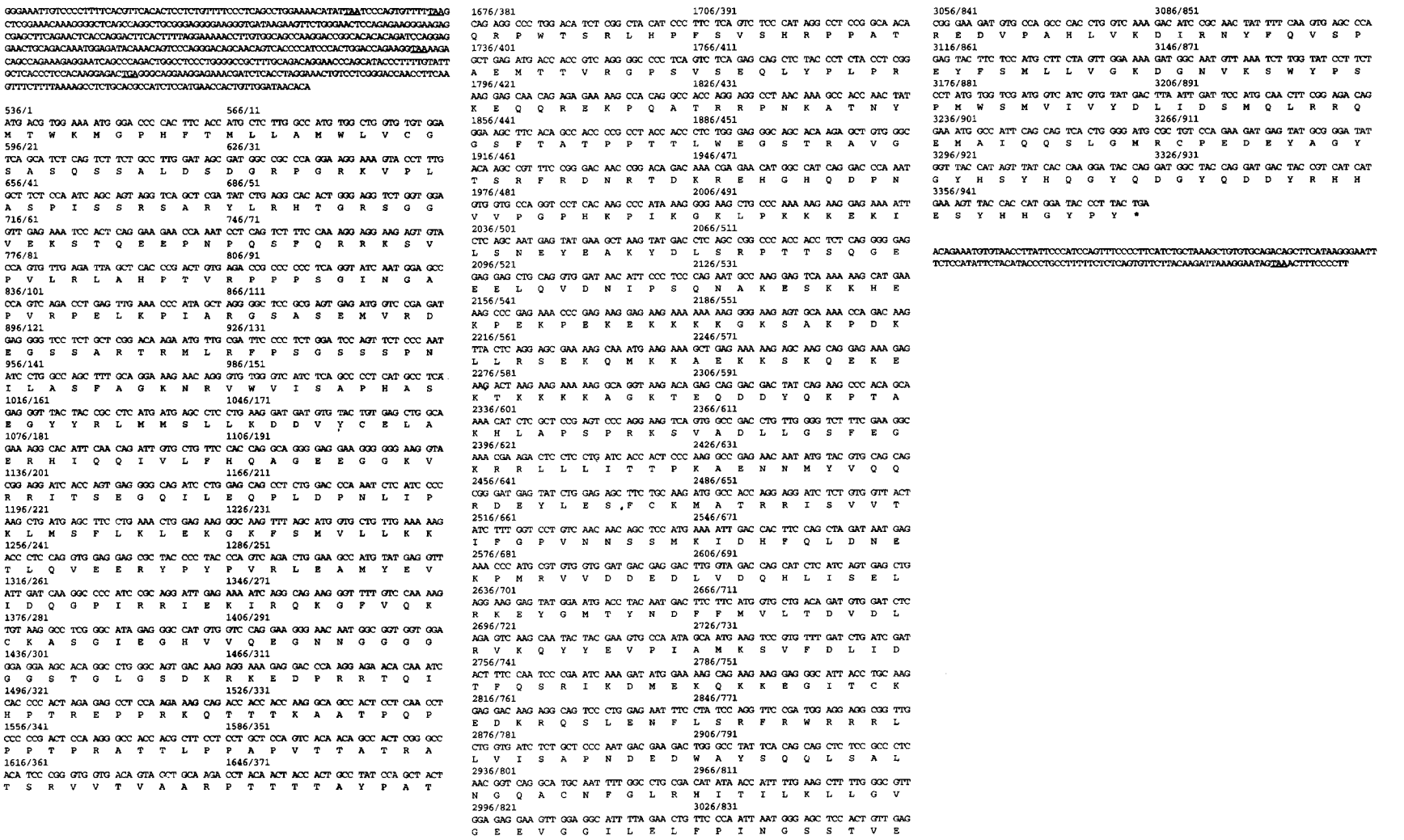
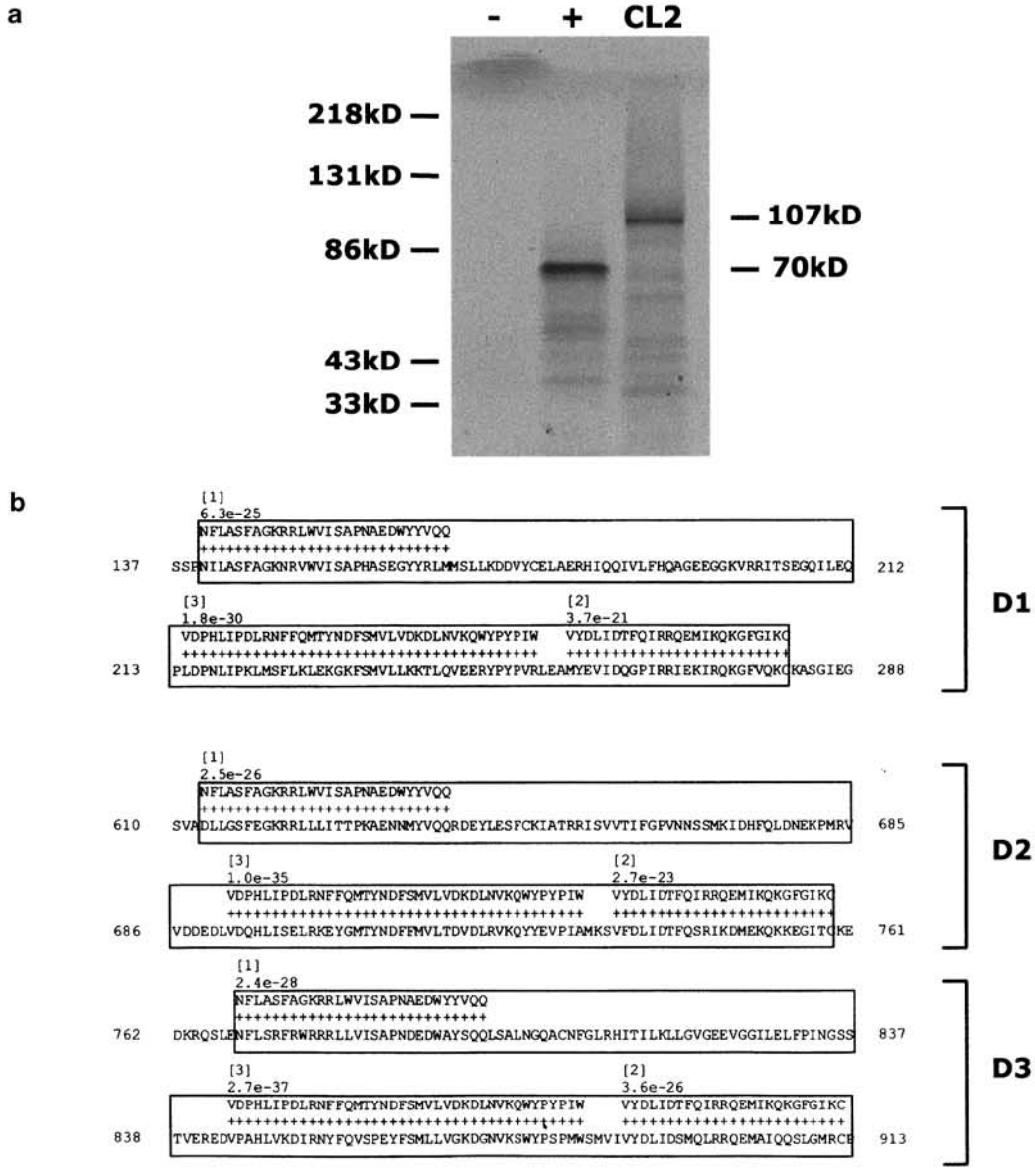


Figure 2 CL2 nucleotide and deduced amino-acid sequences. The CL2 nucleotide sequence (3540 bp) is a composite of the two overlapping cDNAs λCL2-22 (3221 bp) and λCL2-24 (3492 bp). The translated amino-acid sequence (949 aa) of the ORF is given in the single-letter code. The initiating methionine was assigned to the first in-frame ATG sequence. The underlined regions correspond to in-frame stop codons


a
- + CL2
218kD —
131kD —
86kD —
43kD —
33kD —
— 107kD
— 70kD
b
[1]
6.3e-25
NFLASFAGKRRLWVISAPNAEDWYYVQQ
137 SSENILASFAGKNRVWVISAPHASEGYYRLMMSLLKDDVYCELAERHIQQIVLFHQAGEEGGKVRRITSEGQILEQ 212
[3]
1.8e-30
VDPHLIPDLRNFFQMTYNDFSMVLVDKDLNVKQWYPYPIW
213 PLDPNLIPKLMSFLKLEKGKFSMVLLKKTLQVEERYPYPVRLEAMYEVIDQGPIRRIEKIRQKGFVQKCKASGIEG 288
[2]
3.7e-21
VYDLIDTFQIRRQEMIKQKGFGIKC
D1
[1]
2.5e-26
NFLASFAGKRRLWVISAPNAEDWYYVQQ
610 SVADLLGSFEGKRRLLLITTPKAENNMYVQQRDEYLESFCKIATRRISVVTIFGPVNNSSMKIDHFQLDNEKPMRV 685
[3]
1.0e-35
VDPHLIPDLRNFFQMTYNDFSMVLVDKDLNVKQWYPYPIW
686 VDDEDLVDQHLISELRKEYGMTYNDFFMVLTDVDLRVKQYYEVPIAMKSVFDLIDTFQSRIKDMEKQKKEGITCKE 761
[2]
2.7e-23
VYDLIDTFQIRRQEMIKQKGFGIKC
D2
[1]
2.4e-28
NFLASFAGKRRLWVISAPNAEDWYYVQQ
762 DKRQSLENFLSRFRWRRRLLVISAPNDEDWAYSQQLSALNGQACNFGLRHITILKLLGVGEEVGGILELFPINGSS 837
[3]
2.7e-37
VDPHLIPDLRNFFQMTYNDFSMVLVDKDLNVKQWYPYPIW
838 TVEREDVPAHLVKDIRNYFQVSPEYFSMLLVGKDGNVKSWYPSPMWSMVIVYDLIDSMQLRRQEMAIQQSLGMRCE 913
[2]
3.6e-26
VYDLIDTFQIRRQEMIKQKGFGIKC
D3
c
SP
D1
NLS
D2
NLS
D3
d
. . . .10 . . . .20 . . . .30 . . . .40 . . . .50 60
D1 1:NILASFAGKNRVWVISAPHASEGYYRLMMSLLKDDVYCELAERHIQQIVLFHQAGEEGGK: 60
D2 1:DLLGSFEGKRRLLLITTPKAENNMYVQQRDEYL.ESFCKIATRRISVVTIFGPVNNSSMK: 59
D3 1:NFLSRFRWRRRLLVISAPNDEDWAYSQQLSALN.GQACNFGLRHITILKLLGVGEEVGGI: 59
. . . .70 . . . .80 . . . .90 . . . 100 . . . 110 . . . 120
D1 61:VRRITSEGQILEQP......LDPNLIPKLMSFLKLEKGKFSMVLLKKTLQVEERYPYPVR:114
D2 60:IDHFQLDNEKPMRVVDDEDLVDQHLISELRKEYGMTYNDFFMVLTDVDLRVKQYYEVPIA:119
D3 60:LELFPINGSSTVEREDL....VPAHLVKDIRNYFQVSPEYFSMLLVGKDGNVKSWYPSPMW:115
. . . 130 . . . 140 . . . .
D1 114:.LEAMYEVIDQGPIRRIEKIRQKGFVQKC:142
D2 119:.MKSVFDLIDTFQSRIKDMEKQKKEGITC:147
D3 116:SMVIVYDLIDSMQLRRQEMAIQQSLGMRC:144

549 (KKKK), 583 (KKKK), 494 (PKKKEKI), 545 (PEKEKKK), 420 (RKEQQREKPQATRRPNK, bipartite), 537 (KKHEKPEKPEKEKKKKG, bipartite), 569 (KKAEKKSKQEKEKTKKK, bipartite), 573 (KKSKQEKEKTKKKKAGK, bipartite), 608 (RKSVADLLGSFEGKRRL, bipartite) and 763 (KRQSLENFLSRFRWRRR, bipartite). In addition, the CL2 cDNA-derived ORF shows a signal peptide ending at amino acid 24. Based on these results, it is likely that the CL2 gene encodes both nuclear and secreted proteins.

By looking for common motifs in the CL2-encoded protein, we identified a cluster of subsequences (Figure 3, Panel b) occurring three times along the protein (domains) (Figure 3, Panel c). The alignment of the three domains (Figure 3, Panel d) showed an overall identity of 27% and a similarity of 48%.

Screening of the blastn database using the CL2 domains as a probe revealed three proteins whose sushi-repeat motifs are known. The rat *drs* (down-regulated by v-*src*) gene (Pan *et al*., 1996), its human homologue, the *ETX1* gene (or *SRPX*, sushi-repeat-containing protein) (Dry *et al*., 1995) and the human SRPUL gene (sushi-repeat protein upregulated in leukemia) (Kurosawa *et al*., 1999) all share homology with the CL2 domains outside the cysteine-rich sushi-repeat motifs (27% overall identity; 46% overall similarity) (Figure 4). These data indicate that these proteins may be functionally related.

Screening of human genomic sequences available in the GeneBank with the cDNA of CL2 revealed a contig (AC048334) containing the complete ORF of the human CL2 gene. This is located in between chromosomal bands 3q13.2–3q13.3 (confirmed by FISH, not shown) and it is in a tel-5′-3′-cen orientation. The predicted human CL2 amino-acid sequence is 82% identical and 86% similar to that of the rat, indicating an important role of this gene in higher eukaryotics.

Figure 3 Sequence analysis and structure of the CL2 protein. Panel **(a)** *In vitro* transcription–translation. *In vitro* transcription–translation was performed in the presence of [^{35}S]methionine using the TNT Promega kit. A total of 3 μl of the reaction was loaded on a 10% SDS–PAGE and autoradiographed. As expected, the translation product migrates around 107 kDa. The construct pcDNA3-RET/PTC3 (lane +), encoding an unrelated protein of about 70 kDa, was used as positive control for the transcription–translation reaction. Panel **(b)** MAST analysis. Motif alignment and search tool (MAST) analysis of the protein identified three motifs representing the best possible matches among the regions showing sequence similarity: NFLASFAGKRRLWVISAPNAEDWYYVQQ[1], VYDLIDTFQIRRQEMIKQKGFGIKC[2] and VDPHLIPDLRNFFQMTYNDFSMVLVDKDLNVKQWYPYPIW [3]. The number above the aligned sequences indicates the sequence *P*-value, defined as the probability of a random sequence of the same length scoring at least as good as the observed match. Dl, D2 and D3 indicate the assigned protein domains (boxed sequences). Panel **(c)** CL2 protein structure. The organization of CL2 protein is shown schematically. The boundaries of each domain (amino acids 140–281, 612–759 and 768–912, respectively) were selected based on the properties of the sequence and their alignment to other known protein sequences (SRPUL, *ETX1* and *drs*). SP: signal peptide (amino acids 1–24). NLS: nuclear localization signal. Panel **(d)** CL2 domain alignment. Identical amino acids are shaded in darker gray than similar amino acids. Sequence alignments were performed with ClustalW and were displayed with BOXSHADE (http://www.isrec.isb-sib.ch:8080/software/BOX_form.html)

Expression of the CL2 gene in PC Cl 3 cells transformed by other oncogenes

The infection of PC Cl 3 cells with several murine retroviruses exerts different effects on the differentiated and transformed phenotype (Fusco *et al*., 1987; Berlingieri *et al*., 1988, 1993; Santoro *et al*., 1993). While the PC v-*mos* and the PC PyMLV cells were dedifferentiated and tumorigenic, the PC v-*raf*, the PC v-*src* and the PC E1A cells were dedifferentiated but not tumorigenic when injected into nude mice. The PC E1A cells were transformed to an irrefutable neoplastic phenotype after introducing a second oncogene such as the polyoma middle-T antigen or v-*src* genes (Berlingieri *et al*., 1993). These cell lines were analysed for the expression of the CL2 gene. Figure 5 shows that the gene is abundantly expressed in PC E1A cells and PC E1A cells infected with either the polyoma middle-T or the v-*src* oncogenes indicating that the introduction of another transforming oncogene does not modify the expression of the CL2 gene in thyroid cells carrying the E1A gene. Moreover, CL2 was also expressed in the PC v-*raf* cell line, albeit at a lower level than in PC E1A cells. There was no expression in the PC Cl 3 cells infected with the myeloproliferative sarcoma virus (MPSV) carrying the v-*mos* oncogene, with MRSV carrying the v-*src* oncogene, or with polyoma murine leukemia virus (PyMLV), carrying the polyoma middle-T gene.

CL2 cellular localization

To define the intracellular localization of CL2, we constructed an expression vector that contains the entire ORF of CL2 fused in-frame to a gene encoding an enhanced version of the green fluorescent protein (EGFP). This construct was transfected transiently into COS7 cells and monitored for EGFP expression 48 h post-transfection. Expression of EGFP alone resulted in cytoplasmic and nucleoplasmic signals, clearly absent from the nucleoli (Figure 6, Panel a). In contrast, expression of EGFP-CL2 protein resulted in two different patterns of localization. One, observed in most cells, was characterized by a juxtanuclear accumulation of the signal, suggestive of Golgian localization (Figure 6, Panel b). In the remaining 5–10% of the cells, the signal appeared mainly throughout the cytoplasm, with some nuclear–nucleolar staining, heterogeneous in intensity depending on the cell (Figure 6, Panel c).

Tissue distribution and expression

Northern blot analysis of poly(A)$^+$ mRNA from adult human tissues (Figure 7, Panel a) showed two alternative forms of the CL2 transcript (4.2 and 3.8 kb, respectively). The gene expression was very abundant in the heart, barely detectable in brain, liver, spinal cord and lymph node and not detectable in bone marrow and

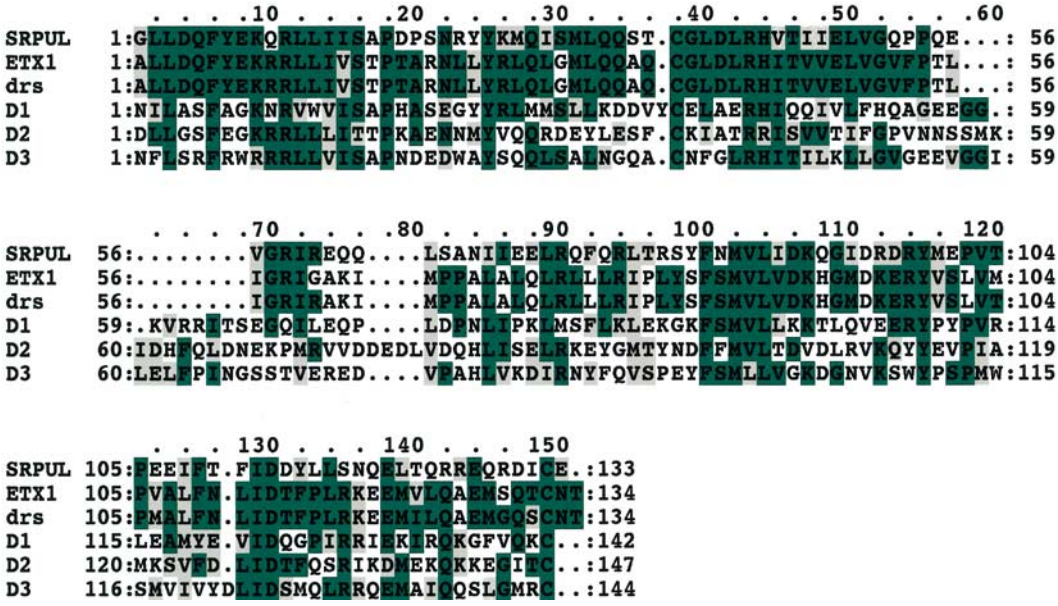


Figure 4 Alignment of CL2 homologues. The rat *drs*, its human homologue, the *ETX1* gene, and the human SRPUL gene all share homology with the CL2 domains outside the cysteine-rich sushi-repeat motifs. Identical and similar amino acids are indicated in green and gray, respectively. Sequence alignments were performed with ClustalW and were displayed with BOXSHADE (http://www.isrec.isb-sib.ch:8080/software/BOX form.html)

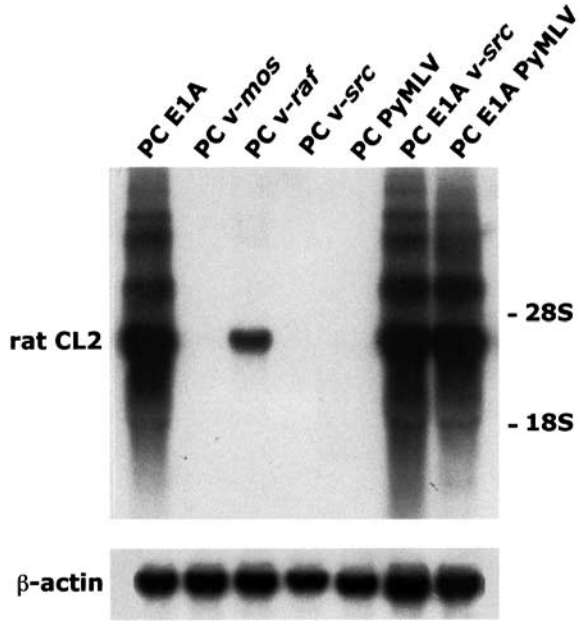


Figure 5 Analysis of CL2 gene expression in PC Cl 3 cells transformed with several oncogenes. In all, 10 μg of total RNA for each cell line were size fractionated on a 1.2% denaturing formaldehyde agarose gel, blotted onto Nylon filters hybond-N and probed with CL2. β-actin was used as control for uniform RNA loading

peripheral blood leukocytes. The larger transcript appeared to be expressed more abundantly than the shorter form. The same pattern of distribution was observed in mouse adult tissues (data not shown). It should be noted that CL2 is expressed as a single transcript in the rat and mouse cells. Finally, analysis of CL2 gene expression at 7, 11, 15 and 17 dpc showed late CL2 expression in mouse embryos (Figure 7, Panel b).

CL2 expression in human thyroid carcinoma cell lines and tumors

To investigate whether CL2 might be involved in human thyroid carcinogenesis, we analysed CL2 expression by RT–PCR in several human thyroid carcinoma cell lines of different histotypes (Figure 8, Panel a). There was no expression in three cell lines originating from papillary carcinomas (B-CPAP, NYM 1 and NPA) and in one originating from an anaplastic carcinoma (ARO). Expression levels comparable to normal thyroid were seen in one cell line from a follicular carcinoma (WRO), in two from papillary carcinomas (TPC-1, FB2) and in two from anaplastic carcinomas (FRO, FB1). We also evaluated CL2 gene expression in biopsies from four goiters and four thyroid adenomas. As shown in Figure 8, Panel b, CL2 gene was highly expressed in both normal thyroid tissues and in the goiters analysed. Significantly, CL2 gene expression was downregulated in three out of the four adenomas. To further investigate CL2 expression in human thyroid tumors, we analysed 14 carcinomas of different hystotypes (12 papillary, one follicular and one anaplastic carcinomas). As shown in Figure 8, Panel c, CL2 expression was very low or absent in the differentiated thyroid carcinomas (13/13) compared with normal tissues and with the anaplastic carcinoma.

Discussion

Here we report the sequence and characteristics of the newly isolated CL2 gene, whose expression is induced in

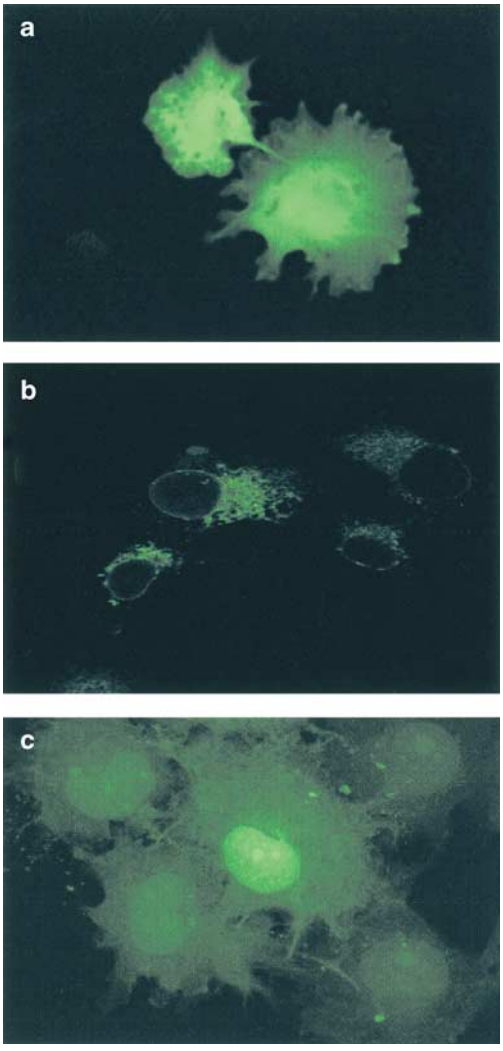


Figure 6 Cellular localization of EGFP-CL2 in COS7 cells. COS7 cells were transiently transfected and monitored for EGFP-CL2 expression 48 h post-transfection. Fluorescence was examined with a Zeiss LSM410 scanning confocal microscope. Panel **(a)** COS7 cells transfected with the EGFP control vector. Panel **(b)** Polar perinuclear accumulation of the EGFP-CL2 signal, suggestive of a Golgian localization. Panel **(c)** The signal is spread throughout the cytoplasm, with some nuclear–nucleolar staining, heterogeneous in intensity depending on the cell

the rat thyroid epithelial cell line PC Cl 3 by the adenovirus E1A gene. In an earlier study, we found that introduction of the adenovirus 5 E1A oncogene into PC Cl 3 cells blocks several differentiated functions and attenuates their dependence on TSH for growth, although they remain unable to grow in semisolid medium and to induce tumors when injected into athymic mice (Berlingieri *et al*., 1993). Hopefully, the identification and characterization of the genes induced by the adenovirus E1A gene in PC Cl 3 cells will shed some light on the mechanisms by which the adenovirus E1A gene exerts its effects on thyroid cells.

The differential screening of a cDNA library prepared from RNA extracted from PC E1A cells led to the isolation of the CL2 gene. CL2 is highly expressed in several cell clones of PC E1A cells, and also in PC cells transfected with the E1A gene deriving from another adenovirus strain, thereby ruling out that induction of this gene by E1A is a cell clonal event. Furthermore, CL2 expression remains high in PC E1A cells carrying an additional oncogene, which indicates that CL2 is important for E1A gene function. An analysis of PC Cl 3 cells transfected with various oncogenes showed that only PC v-*raf* cells express a significant amount of CL2. By activating mitogen-activated protein kinase kinases, raf is a requirement of a key cellular signal pathway (Marshall, 1994; Marais *et al*., 1997). It is tempting to envisage a common mechanism whereby E1A and v-*raf* induce CL2 expression.

A search through protein databases revealed three proteins that have significant homology with the CL2 repeat domains: rat *drs* (Pan *et al*., 1996), its human homologue, *ETX1* (Dry *et al*., 1995) and human SRPUL (Kurosawa *et al*., 1999). These proteins share a cysteine-rich motif, known as a sushi repeat; however, the homology with CL2 extends beyond the sushi-repeat region. In general, conservation between these repeat domains is relatively low, but they do contain stretches of highly conserved sequence motifs. Furthermore, CL2, *drs*, *ETX1* and SRPUL all have a leader peptide at their amino terminal end; hence, they can be secreted. Taken together, these findings indicate that this group of proteins may be functionally related and may constitute a new protein family.

While examining the subcellular distribution of transiently expressed CL2 we noted that in 5–10% of the transfected cells the protein localized to the nucleus/nucleolus. This finding was in keeping with the predicted presence of numerous NLS in the CL2 protein sequence. However, in most cells, the signal accumulated in a juxtanuclear position, which suggests Golgian localization. Also this finding is in keeping with the presence of a leader peptide, typical of secreted proteins. One may gain insight into the stimuli and the mechanism whereby cells produce the secreted or intracellular form of CL2 by looking at already characterized proteins that have the same distribution as CL2. A number of peptide growth factors (βFGF (Bouche *et al*., 1987; Baldin *et al*., 1990; Hill *et al*., 1992), αFGF (Imamura *et al*., 1990), FGF3 (Antoine *et al*., 1997), PDGF (Maher *et al*., 1989), angiogenin (Moroianu and Riordan, 1994) and PTHrP (Henderson *et al*., 1995)) are known to modulate cellular function by a dual mode of action: first, by activating classical signal transduction pathways (secreted form) and, second, by activities within the nucleus (increted form). Furthermore, the localization and, thus, the function of one form can counteract the function of the other.

The CL2 gene is expressed almost ubiquitously in human tissues, which implies that it is involved in the functioning of many organs. Moreover, it is very well conserved during evolution. Therefore, this gene probably plays an important role in higher eukaryotes.

We also analysed CL2 expression in human thyroid carcinoma cell lines and tumors. We chose to investigate thyroid tumors because CL2 was first isolated from

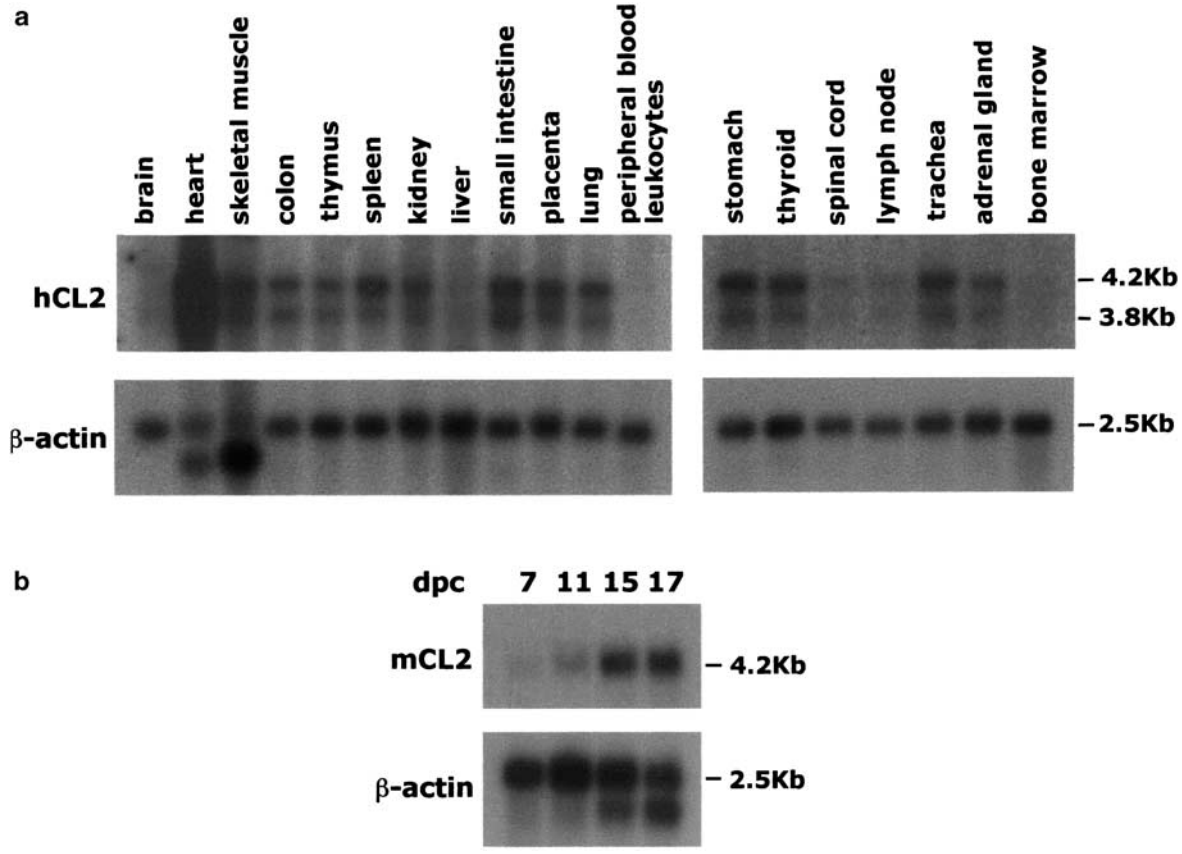


Figure 7 Expression pattern of CL2 mRNA. Two human multiple tissue Northern blots (Panel **a**) and a mouse embryo Northern blot (Panel **b**) were hybridized with a 1.1 kb *Eco*RV/*Hind*III fragment from the pGem3ZCL2 vector. Autoradiograms were exposed for 4 days

thyroid cells. Surprisingly, CL2 expression, clearly detectable in normal human thyroid tissue and in hyproproliferative disorders as goiters, was either very low or absent in some carcinoma cell lines and in most of the tumors, both benign and malignant, analysed, suggesting that the gene plays a tumor-suppressor role in human thyroid carcinogenesis. This hypothesis is further supported by our preliminary results showing a drastic reduction of CL2 gene expression also in ovarian carcinoma cell lines and tissues. Thus, the possibility of CL2 playing a role as tumor suppressor in thyroid as well in other human tumors will warrant further investigation. It will be very interesting to investigate if in human tumors there is LOH on chromosomal bands 3q13.2–3q13.3, where CL2 gene has been mapped.

The finding that CL2, although highly induced by the E1A gene, is indeed downregulated in thyroid human neoplasias may be explained by the proapoptotic effects associated with E1A. In fact, cells stably expressing E1A are 'sensitized' to such apoptotic stimuli as serum depletion, DNA damaging agents, hypoxia, Fas and TNF-α (Evan and Littlewood, 1998). Moreover, E1A-expressing cells possess oncogene-generated activity, that is they activate apoptosis in cell-free systems (Fearnhead *et al*., 1997).

Further studies are necessary to elucidate the role of the CL2 gene product in cell cycle entry, apoptosis and cellular senescence in normal cells as well as in tumors. Transfection experiments of the CL2 gene in the sense and antisense orientation, as well as the creation of deletion mutants, might also provide data about its role as an E1A gene effector and about the mechanisms underlying its cellular destiny.

Materials and methods

Cell cultures

The PC Cl 3 cell line is a thyroid epithelial cell line derived from 18-month-old Fischer rats (Fusco *et al*., 1987). The PC E1A (Berlingieri *et al*., 1993), PC HE4 (Rossini, 1983), PC v-*mos* (Fusco *et al*., 1987), PC v-*raf* (Fusco *et al*., 1987), PC v-*src* (Fusco *et al*., 1987), PC PyMLV (Berlingieri *et al*., 1988), PC E1A v-*src* (Berlingieri *et al*., 1993) and PC E1A PyMLV (Berlingieri *et al*., 1993) cells are described elsewhere. The cell clones analysed in Figure 1, Panel b were obtained transfecting PC Cl 3 cells with the construct pSV2neo13Swt, encoding the E1A 13S gene. At 1 day after the transfection, G418 selection was initiated and continued for 3 weeks. The clones were then expanded and lysed 30 days after the transfection when the mRNA was prepared.

The following human thyroid carcinoma cell lines were used in this study: TPC-1 (Tanaka *et al*., 1987), WRO (Estour *et al*., 1989), NPA (Pang *et al*., 1989), ARO (Pang *et al*., 1989), FRO (Fagin *et al*., 1993), NYM 1 (Zeki *et al*., 1991), FB1 (Fiore

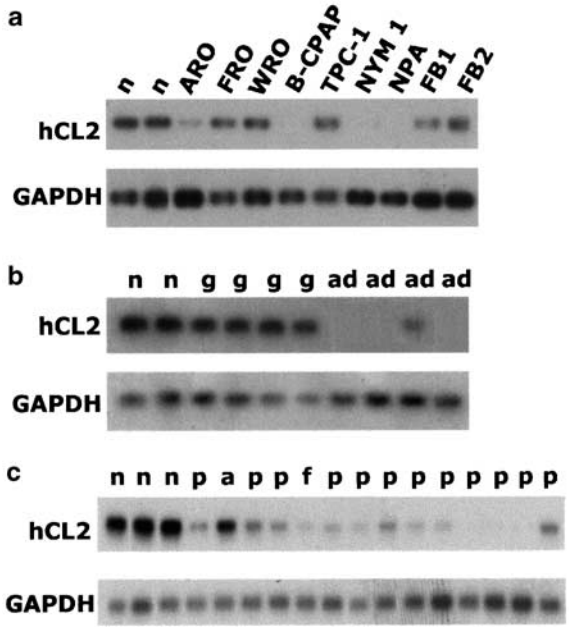


Figure 8 Expression of the CL2 gene in human thyroid carcinoma cell lines (Panel **a**) and tumors (Panels **b** and **c**). CL2 mRNA was amplified by RT–PCR. The RT–PCR products were transferred to a nylon membrane and analysed by hybridization with a [^{32}P]-radiolabeled full-length CL2 cDNA. As an internal control for the amount of RNA, the expression of GAPDH gene was used

et al., 1997), FB2 (Basolo *et al*., 2002) and B-CPAP (Fabien *et al*., 1994). The human cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) containing 10% fetal bovine serum (Flow Laboratories). The normal and infected rat cell lines were cultured in modified F12 medium, supplemented with 5% calf serum (Flow Laboratories) and six growth factors (thyrotropin, hydrocortisone, insulin, transferrin, somatostatin and glycyl-histidyl-lysine; Sigma Chemical Corporation). COS7 (Gluzman, 1981) monkey kidney cells were grown in DMEM supplemented with 10% fetal bovine serum.

PC E1A cell library preparation and library screenings

Oligo(dT)-selected poly(A)$^+$ mRNA was prepared from PC E1A cells using standard methods (Sambrook *et al*., 1989). Purified mRNA (1 μg) was used to prepare double-strand cDNA using the RiboClone cDNA synthesis kit (Promega). This cDNA was ligated to *Eco*RI linkers (Promega), restricted with *Eco*RI and ligated into *Eco*RI digested gt10 arms (Amersham). The ligation was packaged (Gigapack Gold, Stratagene) and 10^6 recombinants obtained. 5×10^4 recombinants were screened using as probes cDNA synthesized from PC E1A or from normal PC Cl 3 cells. For the preparation of the probes, 2 μg of poly(A)$^+$ mRNA extracted from PC Cl 3 or PC E1A cells were reverse-transcribed using an oligo(dT) primer and [α-^{32}P] dCTP (400 Ci/mMol, Amersham) with the RiboClone cDNA synthesis kit; 1×10^6 c.p.m. were used for each 132-mm filter (Schleicher & Schuell). Several clones hybridizing with the labeled PC E1A cDNA, but not with the PC Cl 3 cDNA, were purified. One, CL2, was selected for further study. The CL2 cDNA was then cloned into the plasmid vector pGem3Z (Promega). DNA sequencing on both strands was performed using fluorescently labeled dye-terminator-based sequencing methods on an ABI 377 Prism automated sequencer (Applied Biosystems Inc.).

A rat brain cDNA library constructed in Lambda gt11 vector (Stratagene) was used for the isolation of full-length CL2 cDNA. The library was screened following the protocols provided by the manufacturer.

Sequence analysis

The EST and High Throughput Genomic databases were screened, using the blastn algorithm in the BLAST 2.0 program (Altschul *et al*., 1997) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), with the full-length CL2 cDNA. Computer-based prediction and analysis tools were also used to examine the CL2-deduced protein sequences. SignalIP (http://www.cbs.dtu.dk/services/SignalP) (Nielsen *et al*., 1997) and PSORT II (http://psort.nibb.ac.jp/form2.html) (Nakai and Kanehisa, 1992) programs were used to predict subcellular localizations. To discover motifs in the CL2 protein sequence, we used the Motif Alignment and Search Tool (MAST, version 3.0) (Bailey and Gribskov, 1998) available on-line at the http://meme.sdsc.edu/meme/website/mast-intro.html site. Protein sequences were aligned using ClustalW (Thompson *et al*., 1994) and shaded using the Boxshade program (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html).

In vitro *transcription–translation*

Using the pGem3ZCL2 vector as a template, the entire CL2 ORF was amplified by PCR with the oligonucleotides 5′ GCCGAATTCAGGATAACACAATGACGTG 3′ and 5′ TGCGGTCGACTGTTCAGTAAGGGTATCC 3′ carrying a 5′ *Eco*RI and a 3′ *Sal*I restriction site. After digestion, this fragment was subcloned into pcDNA3 vector (Invitrogen) and *in vitro* transcription–translation was performed with [^{35}S]methionine using the TNT Promega kit (Promega). In total 3 μl of the reaction was resolved by 10% SDS–PAGE and subjected to autoradiography. The construct pcDNA3-RET/PTC3 (Melillo *et al*., 2001), encoding for an unrelated protein of about 70 kDa, was used as a control.

RNA extraction and Northern blot analyses

RNA was purified from cultured cells and several types of tumoral tissue by a modification of the guanidine thiocyanate method (RNAFast). For the Northern blot procedure, 10 μg of total RNA was size fractionated on a denaturing formaldehyde agarose gel and blotted onto Nylon filters hybond-N (Amersham). A 1.2 kb *Eco*RV/*Hind*III fragment from the pGem3ZCL2 vector was used as a probe to assay the CL2 gene expression levels, while a mouse β-actin probe (Tokunaga *et al*., 1986) was used to ascertain equal RNA loading. A *Cla*I/*Xba*I fragment of the E1A adenovirus 5 gene was used as a probe to assay E1A gene expression levels. Probes were labeled with the random oligonucleotide primer kit (Amersham). The tissue-specific expression of the CL2 mRNA and its embryonic expression were determined using two human multiple tissue Northern blots and a mouse embryo panel (Clontech), according to the manufacturer's protocol.

RT–PCR analysis

A total of 2 μg of DNAse-digested total RNA were reverse transcribed using random hexamers as primers (100 mM) and 12 units of AMV reverse transcriptase (Promega). The cDNA obtained was amplified with primers specific for CL2 (FW 5′ AAGACTGGGCCTATTCACAG 3′, RV 5′ CCATCCTGGTATCCTTGGTG 3′) in a 25 μl reaction mixture containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 mM of each primer, 1 unit

Taq DNA polymerase (Perkin–Elmer). After 20 amplification cycles (95°C: 1 min; 55°C: 30 s; 72°C: 30 s) 10 μl of the products were separated on a 1% agarose gel, blotted onto hybond-N nylon filters (Amersham) and hybridized with a [^{32}P]-radiolabeled full-length CL2 cDNA. Expression of the GAPDH gene was used as internal control for the amount of RNA (GAPDH FW 5′ ACATGTTCCAATATGATTCC 3′; GAPDH RV 5′ TGGACTCCACGACGTACTCA 3′). It was amplified in the same PCR conditions as the CL2 gene.

Cellular localization study

An EGFP fusion construct was made for the analysis of the cellular localization of CL2 protein. The oligonucleotides 5′ GCCGAATTCAGGATAACACAATGACGTG 3′ and 5′ CGGGGTACCAAGTAAGGGTATCCATGGTG 3′ were used to obtain a PCR product carrying a 5′ *Eco*RI and a 3′ *Kpn*I restriction site. This PCR product was digested and ligated into pEGFP-N1 (Clontech) to create an in-frame C-terminal fusion with EGFP.

For transient expression of this construct, COS7 cells were grown on glass coverslips. At a 50–80% confluency, cells were transfected with 4 μg of pEGFP-N1 CL2 DNA or with the control vector using Fugene (Roche). After 2 days of expression, the cells were washed with PBS and fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min at room temperature. Cells were then washed twice with PBS, and coverslips were mounted in Fluoromount-G (Southern Biotech Inc.). GFP staining was examined using a Leica TCS SP II scanning confocal microscope equipped with an argon–krypton laser.

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