Induction of apoptosis by the mouse *Nedd2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1β-converting enzyme

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By subtraction cloning we previously identified a set of mouse genes (named *Nedd1* through *Nedd10*) with developmentally down-regulated expression in brain. We now show that one such gene, *Nedd2*, encodes a protein similar to the mammalian interleukin-1β-converting enzyme (ICE) and the product of the *Caenorhabditis elegans* cell death gene *ced-3* (CED-3). Both ICE and CED-3 are known to encode putative cysteine proteases and induce apoptosis when overexpressed in cultured cells. Overexpression of *Nedd2* in cultured fibroblast and neuroblastoma cells also resulted in cell death by apoptosis, which was suppressed by the expression of the human *bcl-2* gene, indicating that *Nedd2* is functionally similar to the *ced-3* gene in *C. elegans*. We also show that during embryonic development, *Nedd2* is highly expressed in several types of mouse tissue undergoing high rates of programmed cell death such as central nervous system and kidney. Our data suggest that *Nedd2* is an important component of the mammalian programmed cell death machinery.

[*Key Words*: Programmed cell death; development; ICE; CED-3; cysteine protease]

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The development of multicellular organisms requires several active gene-regulated processes, such as cell proliferation, differentiation, and death. Although much effort has been devoted to the understanding of the molecular events controlling cell proliferation and differentiation, the importance of cell death has become apparent only recently (for review, see Williams and Smith 1993). Extensive physiological or programmed cell death (PCD) occurs in many animal tissues during development (Glucksmann 1951). PCD is necessary to eliminate unwanted cells and achieve homeostasis (for review, see Ellis et al. 1991; Raff 1992; Vaux et al. 1994). The term apoptosis is often used to describe the active death of mammalian cells and is characterized by a distinct set of events such as plasma membrane blebbing, nuclear condensation, loss of cell volume, and fragmentation of DNA at nucleosomal intervals (Kerr et al. 1972; Wyllie et al. 1980). The best characterized genetic system of PCD is the worm *Caenorhabditis elegans* in which 131 cells undergo PCD during development (Sulston and Horvitz 1977; Sulston et al. 1983). There are 14 genes identified that function in different steps of PCD (for review, see Ellis et al. 1991). Among these, *ced-3* and *ced-4* are required for cell death to occur and, consequently, mutants lacking either of these genes have extra cells (Ellis and Horvitz 1986). The *ced-9* gene antagonizes the function of *ced-3* and *ced-4* by protecting cells from PCD (Hengartner et al. 1992). In the mutants lacking *ced-9*, most of the cells arrest early in the development, and this mutation can be suppressed completely by a second mutation in *ced-3* or *ced-4* suggesting that *ced-3* and *ced-4* are located downstream of *ced-9* (Hengartner et al. 1992). *ced-9* has been shown to encode a protein similar to the product of the mammalian proto-oncogene *bcl-2* (Hengartner and Horvitz 1994). The function of *ced-9* mutation can be partially restored by the expression of the human *bcl-2* gene, indicating that the pathways of PCD are similar in *C. elegans* and mammals

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(Vaux et al. 1992; Hengartner and Horvitz 1994). The *bcl-2* gene, originally cloned from the breakpoint of a t(14;18) chromosomal translocation in human B-cell lymphomas (Cleary et al. 1986; Tsujimoto and Croce 1986), is known to prevent in vitro and in vivo apoptosis of a wide variety of cells (for review, see Korsmeyer 1992).

The *ced-3* and *ced-4* genes are expressed at high levels during the embryonic development of *C. elegans* when most of the cell death takes place (Yuan and Horvitz 1990, 1992). The *ced-4* gene encodes a novel 63-kD protein with a putative EF-hand domain, suggesting that it may be a calcium-binding protein (Yuan and Horvitz 1992). Recently *ced-3* gene has been cloned, and its product (CED-3) shown to be similar to mammalian interleukin-1β-converting enzyme (ICE) (Yuan et al. 1993). In cultured cells the overexpression of both *ced-3* and ICE was shown to induce apoptosis that is blocked by *bcl-2* (Miura et al. 1993). Microinjection of ICE cDNA expression vectors into dorsal root ganglia neurons also results in cell death (Gagliardini et al. 1994). This provides further evidence that at least one of the pathways regulating PCD is essentially conserved between *C. elegans* and mammals.

We are interested in genes that are expressed in a regulated fashion during the development of the mammalian central nervous system (CNS). As most of the neurogenesis takes place during embryonic development of mammals, these genes are expected to play a role in the development and differentiation of the CNS. To identify such genes, we subtracted a cDNA library prepared from mouse neural precursor cells (NPCs) isolated from the neural tube at embryonic day 10 (E10) (Kitani et al. 1991) with mRNA isolated from postnatal and adult brain (Kumar et al. 1992). Ten independent partial cDNA clones were isolated by use of this approach, and the genes representing these clones were named *Nedd* [for NPC-expressed, developmentally down-regulated (genes)] (Kumar et al. 1992). As expected, the expression of these genes is much higher in developing CNS than fully differentiated adult brain (Kumar et al. 1992). Among the *Nedd* genes characterized so far, *Nedd1* encodes a protein with homology to the GTP-binding protein β-subunit and has strong growth-suppressive activity in cells of neuronal lineage (Kumar et al. 1994); *Nedd3* encodes an evolutionarily conserved novel GTP-binding protein of unknown function (Sazuka et al. 1992a,b; Kumar et al. 1993a), whereas *Nedd8* encodes a ubiquitin-like protein (Kumar et al. 1993b). From these studies, it appears that different *Nedd* genes identified by subtraction cloning may be involved in various aspects of development-related cellular processes. Because extensive cell death occurs during the development of the nervous system (for reviews, see Cowan et al. 1984; Davies 1987; Oppenheim 1991), it is also possible that some of the *Nedd* genes may play a role in the regulation of PCD. In this study we have characterized the *Nedd2* gene, which encodes a protein (Nedd2) similar to the product of the *C. elegans* cell death gene *ced-3* and the mammalian ICE. We show that like ICE and CED-3, overexpression of *Nedd2* also induces apoptosis in cultured neuroblastoma and fibroblast cells and this apoptosis can be inhibited by the expression of *bcl-2*. *Nedd2* is strongly expressed in various embryonic tissues during development when most cell death occurs. Thus, this gene is likely to play a general role in the regulation of apoptosis in mammalian cells.

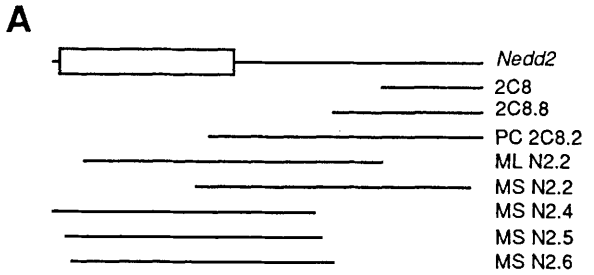
Results

Cloning of Nedd2 *cDNA*

By use of the original *Nedd2* cDNA clone representing the 3′ end of the mRNA (Kumar et al. 1992) we isolated further 5′ clones, which were in turn utilized to obtain near full-length cDNA sequence (Fig. 1A). The sequence compiled from these clones represents 3392 nucleotides excluding the poly(A) stretch and contains a single open reading frame (ORF) starting with a putative initiation codon at nucleotide number 7 (Fig. 1B). The length of the cDNA sequence matches well with the predicted size of the *Nedd2* mRNA (see below). Although the reading frame is open at its 5′ end, the first in-frame ATG is in a good context for translation initiation (Kozak 1986). A second in-frame ATG located 57 nucleotides downstream is also in good context for translation initiation. The ORF ends at nucleotide residue 1362 and is followed by a long 3′-untranslated region (1363–3392). The ORF can encode a protein of 451 amino acid residues with a predicted relative molecular mass of 50,547. Overall, the putative Nedd2 protein is hydrophobic in nature, consisting of 46% hydrophobic, 24% neutral, and 30% hydrophilic residues. There is no potential transmembrane domain in the protein; however, the sequence contains a potential myristylation site at amino acids 341–346 (Fig. 1B). The Asn residue at position 335 and the Tyr residue at position 434 are potential glycosylation and phosphorylation sites, respectively (Fig. 1B). The translation of mRNA prepared in vitro from the three independent cDNA clones resulted in protein products of expected sizes (data not shown). The longest 5′ clone containing the first ATG at position 7 (MS N2.4) gave rise to a major product of 51 kD, whereas two other clones that start 29 (MS N2.5) and 59 (MS N2.6) nucleotide residues farther downstream generated slightly smaller products, which presumably start at the second in-frame ATG initiation codon (data not shown). Minor products of ~45, 28, and 19 kD were also evident in all cases (data not shown).

Nedd2 *encodes a protein similar to CED-3 and ICE*

By use of our previously unpublished *Nedd2* sequence in the data base (accession no. D10713), Yuan et al. (1993) have suggested the potential homology between CED-3, ICE, and Nedd2 proteins. The 5′ region of this sequence had probably arisen from some cloning artifacts (see Material and methods for details); therefore, the deduced product lacked the amino-terminal 322 residues of the putative Nedd2 protein. Further cloning and sequencing (Fig. 1) revealed that *Nedd2* cDNA encodes a protein



B

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CTGGAAATGGCGGCGCCGAGCGGGAGGTCGCAGTCCTCCCTGCACAGGAAGGGGCTGATG   60
      M  A  A  P  S  G  R  S  Q  S  S  L  H  R  K  G  L  M     18
GCGGCTGACAGGAGGAGCAGGATTTTGGCAGTGTGTGGAATGCATCCTGACCACCAGGAA  120
A  A  D  R  R  S  R  I  L  A  V  C  G  M  H  P  D  H  Q  E     38
ACACTGAAAAAGAATCGAGTGGTGCTGGCCAAGCAGCTGCTGCTGAGCGAGCTGTTAGAA  180
T  L  K  K  N  R  V  V  L  A  K  Q  L  L  L  S  E  L  L  E     58
CACCTCCTAGAGAAGGACATTATCACTTTGGAAATGAGGGAGCTCATCCAGGCCAAAGGG  240
H  L  L  E  K  D  I  I  T  L  E  M  R  E  L  I  Q  A  K  G     78
GGCAGTTTCAGCCAGAATGTGGAACTCAACCTGCTGCCAAAGAGAGGACCCCAGGCTTTT  300
G  S  F  S  Q  N  V  E  L  N  L  L  P  K  R  G  P  Q  A  F     98
GATGCCTTCTGTGAAGCCCTGCGGGAGACCAGGCAGGGTCACTTGGAAGACTTACTGCTC  360
D  A  F  C  E  A  L  R  E  T  R  Q  G  H  L  E  D  L  L  L    118
ACAACCCTCTCAGATATTCAGCACGTACTCCCACCGTTGAGCTGTGACTATGACACAAGT  420
T  T  L  S  D  I  Q  H  V  L  P  P  L  S  C  D  Y  D  T  S    138
CTCCCTTTCTCGGTGTGTGAGTCCTGCCCTCCTCACAAGCAGCTCCGCCTATCCACAGAT  480
L  P  F  S  V  C  E  S  C  P  P  H  K  Q  L  R  L  S  T  D    158
GCTACGGAACACTCCTTAGATAATGGTGATGGTCCTCCCTGTCTTCTGGTGAAGCCATGC  540
A  T  E  H  S  L  D  N  G  D  G  P  P  C  L  L  V  K  P  C    178
ACTCCTGAGTTTTACCAGGCACACTACCAGCTGGCCTATAGGTTGCAATCTCAGCCCCGT  600
T  P  E  F  Y  Q  A  H  Y  Q  L  A  Y  R  L  Q  S  Q  P  R    198
GGCTTGGCACTGGTGCTGAGCAATGTGCACTTCACTGGAGAGAAAGACCTGGAATTCCGC  660
G  L  A  L  V  L  S  N  V  H  F  T  G  E  K  D  L  E  F  R    218
TCTGGAGGGGATGTGGACCACACTACTCTAGTCACCCTCTTCAAGCTTTTGGGCTACAAT  720
S  G  G  D  V  D  H  T  T  L  V  T  L  F  K  L  L  G  Y  N    238
GTCCATGTGCTACATGACCAGACCGCACAGGAAATGCAAGAGAAACTTCAGAATTTTGCA  780
V  H  V  L  H  D  Q  T  A  Q  E  M  Q  E  K  L  Q  N  F  A    258
CAGTTACCTGCACACCGGGTCACAGACTCCTGCGTAGTGGCACTCCTCTCACATGGTGTG  840
Q  L  P  A  H  R  V  T  D  S  C  V  V  A  L  L  S  H  G  V    278
GAAGGTGGCATCTATGGTGTAGATGGCAAACTGCTTCAGCTCCAAGAGGTTTTTCGACTT  900
E  G  G  I  Y  G  V  D  G  K  L  L  Q  L  Q  E  V  F  R  L    298
TTTGACAATGCTAACTGTCCAAGTCTACAGAACAAGCCAAAAATGTTCTTCATCCAAGCA  960
F  D  N  A  N  C  P  S  L  Q  N  K  P  K  M  F  F  I  Q  A    318
TGTCGTGGAGATGAGACAGATAGAGGTGTCGACCAGCAAGATGGAAAGAACCACACACAA 1020
C  R  G  D  E  T  D  R  G  V  D  Q  Q  D  G  K  [N]  H  T  Q   338
TCCCCTGGATGTGAGGAGAGTGATGCTGGCAAAGAGGAGTTGATGAAGATGAGACTGCCT 1080
S  P  G  C  E  E  S  D  A  G  K  E  E  L  M  K  M  R  L  P    358
ACTCGCTCAGACATGATATGTGGCTATGCTTGCCTTAAAGGTAATGCTGCCATGCGGAAC 1140
T  R  S  D  M  I  C  G  Y  A  C  L  K  G  N  A  A  M  R  N    378
ACCAAACGGGGTTCCTGGTACATTGAGGCCCTCACTCAGGTGTTCTCTGAAAGAGCTTGT 1200
T  K  R  G  S  W  Y  I  E  A  L  T  Q  V  F  S  E  R  A  C    398
GACATGCACGTGGCCGACATGCTTGTTAAGGTGAATGCCCTTATCAAGGAGCGTGAAGGC 1260
D  M  H  V  A  D  M  L  V  K  V  N  A  L  I  K  E  R  E  G    418
TATGCCCCTGGCACAGAATTCCACCGATGCAAGGAGATGTCTGAGTACTGTAGTACTCTG 1320
Y  A  P  G  T  E  F  H  R  C  K  E  M  S  E  (Y)  C  S  T  L  438
TGCCAGCAACTCTACCTGTTCCCAGGCTACCCACCCACGTGATGCCGCCTGCTATTCCTG 1380
C  Q  Q  L  Y  L  F  P  G  Y  P  P  T  *                     451
CTGTTGGAGGCCACTGGACCACTGGGGGCACAATGGAGACTTCTCTTCAGAATGGTTTTT 1440
GTTCTGTCTACCCTCTCAGGGATATGAGATTCTCCCAGGCTTGTTTCCTGTCAGCCATCT 1500
CTGTCTTTGGGTATGAAACATAAGGATGGCTCCTCCGGTGTCGTGTTCTCTACCTATAGA 1560
GCCAGCTCTGAATGGATGTGTTACCAGAAGCATTTTAGCTACAGCCTAGAAAATGACATT 1620
GTGAACACAGTATTATTGTGGGAAGAGGGCATTTGGATTTCTCAATGTTTGTGATATTTT 1680
TGTTCCCAAGGCATCTTAGGAGTACTTGGATCATAGCTTTTTTTTTTTTTCCTAAATCAG 1740
TTAAGGAGTCTCAGAGATCATCTCCTTTTTTTTCCATATCTACAACCTCATTTTTCCCAC 1800
AGTGGAGATTTGGAAGATGTCCCAATTTAATGTAGGTGTTTTCATCTGTCATGAAGGGAC 1860
AGATGAGATCCTACTACTTGCGAAGTTTCTATGCATACCTTTAAGTTCAGGCCCTAGGTG 1920
AAGGACAGTCCCTCAGCCTTTCCATTGGTTCCTTTGTGTTCAGTGCACCCAGCCTTTGAA 1980
CAGAGCCTAGGGTCTGTATGCCATGACACTGGAAGTCATAGAAATTTCCCTGGTCATGCT 2040
TTGTTTGAACTGTCACTGAATGAACCTTATCGGGCATAACTACATGAAAATGCAGTGACA 2100
GCTGAGTGTGCTGTGTCTCACACTATCACCCGTCATCAGGATGTCTCTCCTTCCTTACTG 2160
TGGCTTCTGCATGCACTTACACTGTACTTGACGGCTGGCCTCCAGGGTCTCTCTTGCTTT 2220
GTACTGGTTCCCCTCTTTACCTTCACCATTCGCTGCTTCTGCCAAGTCTGTGAAGCCGTC 2280
CTTTGTAGGATGTTTCTTGCCACTTACGCTGTACTGTAGTTGCTTATTCTTTCTGCCTTC 2340
TGCTTCAGCGTGAGGCTTCTTTGGTTTTCTGTGGCAGCGTCTCCCTTCTCATTGTTTCTC 2400
TGTGTTTTAGTGGGGATAGTACCATATGTGATATAACCTAGAAGCACTTGTCTCTGCTCT 2460
TATGAAACTTGCTTATTCTTGAAAACCTTCTGCATTTCCATTTTTTCCTCTCTTCCAATT 2520
TATTCTCCATGTAACAGAGTAGTTTGGTTTTTAAAATATCTGGTGATGTCATTCTCTTGC 2580
TTAGAACACTAGCTTCCTGTTACGCTTCATCTAAAATGCAAATTCTTACACCCAGCTTAC 2640
GAGATCTGGCTCATACCTTCCCTTTGGATCTCATTAAATGGTGATTTCTCACTATGCTCC 2700
AGCCCCTCTTAGGTCCTCATCTCCGTCTTGCAGGTGTTCTGAACTCTCCTTTGGCTAGTC 2760
TCTGATTTTTGAGTCTGGCGGAGGCCTCTTGACCATGCTGCCCATGCTGTCTACTGTGCC 2820
TCCTTATGAGGGCATCATGTTGGTCTCTGTTGTGCTTACTGCAGGCTGTAATGGCACTTT 2880
TGCTTGTTTCACTTGTTCCCTCTGAGGCTGAATGCTCCAAGAGAGTGGGAACTGTGCTTC 2940
TTACTTACTGATATCCAGTAACTGGCACTTACTAGGTCTTCATGAATGTTTCCTGAGTAA 3000
AGGAAGGAGACCAGCAGCTAACCTTAGTTAGAGCCTACCTTTTGCAGTTTCTAAATTGCT 3060
ATTATAGTGTACAGTTCAATTAGTATATGGGTTTTTTTTTCCAGGTGTTTTATTTTTATC 3120
CACTGTTTTGTTGTTGTTTTTTTATATTTTCTACATATCACGTGTGAGACAACTTCTTTC 3180
ACATCTCCATAGTGCCCAGCAAATTTGAGGCCTATGGTAGTTGAGGTGCTCAACTAATGT 3240
TTGTTGTATGAACCAAGTGGTTTGAAGACTTGCTGCCAAATTCTGCCTTTTGGGTCAGTA 3300
TAGGATGCATAAGTGGTAGAATCTTCACACTTCCCACTGCCAAGATTTTGTATTGCCATC 3360
AGGTGCCAAATAAATGTTGATACTTATTACTG-(poly A)                    3392
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similar to CED-3 and ICE. A protein sequence alignment of Nedd2, mouse and human ICE, and CED-3 is shown in Figure 2 (below). The Nedd2 protein shows 29% identity (52% similarity) with human and mouse ICE, and 31% identity (55% similarity) with the CED-3 protein (Fig. 2). As ICE and CED-3 are ~29% identical to each other, Nedd2 may be a closer relative of CED-3 than ICE; however, like ICE, Nedd2 protein lacks the serine-rich region present between amino acids 107 and 205 of CED-3 (Yuan et al. 1993). The overall BLAST scores for Nedd2/CED-3, Nedd2/human ICE, and Nedd2/mouse ICE were 156, 136, and 125, respectively. There are 53 residues that are identical among all four proteins (Fig. 2). The amino acids affected in seven of the eight *ced-3* missense mutations (Yuan et al. 1993) are conserved in Nedd2 protein (Fig. 2).

The highest homology between the four proteins lies in their carboxy-terminal half (from amino acid residue 218 of Nedd2). The sequence QACRG containing the active Cys residue required for the proteolytic activity of ICE (Thornberry et al. 1992) is completely conserved among all four proteins. ICE is composed of two subunits P10 and P20, which are thought to originate by self cleavage at Asp residues at positions 103, 119, 297, and 316, respectively, of human ICE (Thornberry et al. 1992). Nedd2 protein contains several Asp residues in the presumptive amino and carboxyl cleavage regions, at least one of which is conserved with respect to ICE (Fig. 2).

Expression of Nedd2 *mRNA is down-regulated during development*

Expression of *Nedd2* mRNA was examined by Northern blot analysis of RNA isolated at various developmental stages (Fig. 3). By use of poly(A)$^+$ RNA from embryonic heads at postcoital days 10–13 (E10–E13) and brain tissues from other stages of development, we detected a single transcript of ~3.5 kb in size (Fig. 3A). As reported earlier (Kumar et al. 1992), the expression of this transcript was down-regulated during development, and only weak hybridization signals are seen for adult brain samples (Fig. 3A). From densitometric analyses we estimate that the signals at E10 are ~30 times stronger than the signals for adult brain (data not shown). Northern analysis of poly(A)$^+$ RNA isolated from total embryos at various stages of development showed a similar pattern, that is, the expression was highest around stages E10–E14 (Fig. 3B). These data show that high *Nedd2* expres-

Figure 1. Cloning and sequencing of *Nedd2* cDNA. (*A*) Structure of the various overlapping cDNA clones isolated from several cDNA libraries and used for sequence analysis. Only the clones characterized in detail are shown. The open box indicates the longest reading frame in the combined *Nedd2* cDNA sequence. (*B*) Nucleotide and predicted amino acid sequence of *Nedd2*. The polyadenylation signal is double underlined. In the protein sequence, a putative myristylation site is underlined, a possible Asn glycosylation site is boxed, and a possible Tyr phosphorylation site is encircled.

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hICE                                MADKVLKEKRKLFIRS.....MGE    19
mICE                                MADKILRAKRKQFINS.....VSI    19
CED-3                        MMRQDRRSLLERNIMMFSSH.............  20
Nedd2   MAAPSGRSQSSLHRKGLMAADRRSRILAVCGMHPDHQETLKKNRVVLAKQ     50

hICE    GTINGLLDELLQTRVLNKEEMEKVKRENATVMDKTRALIDSVIPKGAQAC     69
mICE    GTINGLLDELLEKRVLNQEEMDKIKLANITAMDKARDLCDHVSKKGPQAS     69
CED-3   LKVDEILEVLIAKQVLNSDNGDMIN.SCGTVREKRREIVKAVQRRGDVAF     69
Nedd2   LLLSELLEHLLEKDIITL.EMRELIQAKGGSFSQNVELNL.LPKRGPQAF     98

hICE    Q.ICITYICEEDSYLAGTLG............................      88
mICE    Q.IFITYICNEDCYLAGILE ............................      88
CED-3   DAFYDALRSTGHEGLAEVLEPLARSVDSNAVEFECPMSPASHRRSRALSP    119
Nedd2   DAFCEALRETRQGHLEDLL..............................      117

hICE    ................................................       88
mICE    ................................................       88
CED-3   AGYTSPTRVHRDSVSSVSSFTSYQDIYSRARSRSRSRALHSSDRHNYSSP    169
Nedd2   ..................LTTLSDI........................      124

hICE    ...LSADQTSGNYLNMQQSQGVLSSFPAPQAVQQNPAMPTSSGSEGNVKL     135
mICE    ...LQSAPSAETFVATEDSKGGHPSSSETKE.EQNKEDGTFPGLTGTLKF     134
CED-3   PVNAFPSQPSSANSSFTGCSSLGYSSSRNR FSKASGPTQYIFHEEDMN.    217
Nedd2   .QHVLPPLSCDYDTSLP..FSVCESCPPHKQLRLSTDATEHSLDNGDGPP    171

hICE    CSLEEA..QRIWKQKSAEIYPIMDKSSRTRLALIICNEEF...DSIPRRT     180
mICE    CPLEKA..QKLWKENPSEIYPIMNTTTRTRLALIICNTEF...QHLSPRV     179
CED-3   .FVDAPTISRVFDE..KTMYRNF.SSPR.GMCLIINNEHF...EQMPTRN    259
Nedd2   CLLVKPCTPEFYQAHYQLAYRLQ.SQPR.GLALVLSNVHFTGEKDLEFRS    219

hICE    GAEVDITGMTMLLQNLGYSVDVKKNLTASDMTTELEAFAHRPEHKTSDST     230
mICE    GAQVDLREMKLLLEDLGYTVKVKENLTALEMVKEVKEFAACPEHKTSDST     229
CED-3   GTKADKDNLTNLFRCMGYTVICKDNLTGRGMLLTIRDFAKHESH..GDSA    307
Nedd2   GGDVDHTTLVTLFKLLGYNVHVLHDQTAQEMQEKLQNFAQLPAHRVTDSC    269

hICE    FLVFMSHGIREGICGKKHSEQVPDILQLNAIFNMLNTKNCPSLKDKPKVI     280
mICE    FLVFMSHGIQEGICGTTYSNEVSDILKVDTIFQMMNTLKCPSLKDKPKVI     279
CED-3   ILVILSHGEENVIIGVD.....DIPISTHEIYDLLNAANAPRLANKPKIV    352
Nedd2   VVALLSHGVEGGIYGVD.....GKLLQLQEVFRLFDNANCPSLQNKPKMF    314

hICE    IIQACRGDSPGVVWFK.QSVGVSGNLSLPTTEEFEDQAI...........     318
mICE    IIQACRGEKQGVVLLK.DSVRDSEE.DFLTDAIFEDDGI...........     316
CED-3   FVQACRGERRDNGFPVLDSVDGVPAFLRRGWDNR.DGPLFNFLGCVRPQV    402
Nedd2   FIQACRGDETDRG...VDQQDGKNHTQSPGCEES.DAGKEE LM......     353

hICE    ....KKAHIEKDFIAFCSSTPDNVSWRHPTMGSVFIGRLIEHMQEYACSC     364
mICE    ....KKAHIEKDFIAFCSSTPDNVSWRHPVRGSLFIESLIKHMKEYAWSC     362
CED-3   QQVWRKKPSQADILIAYATTAQYVSWRNSARGSWFIQAVCEVFSTHAKDM    452
Nedd2   ...KMRLPTRSDMICGYACLKGNAAMRNTKRGSWYIEALTQVFSERACDM    400

hICE    DVEEIFRKVRFSFEQPDGRAQMPTTERV........TLTRCFYLFPGH       404
mICE    DLEDIFRKVRFSFEQPEFRLQMPTADRV........TLTKRFYLFPGH       402
CED-3   DVVELLTEVNK..KVACGFQTSQGSNILKQMPEMTSRLLKKFYFWPEARNSAV 503
Nedd2   HVADMLVKVNALIKEREGYAPGTEFHRCKEMSEYCSTLCQQLYLFPGYPPT   451
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Figure 2. Putative Nedd2 protein is similar to CED-3 and ICE. An amino acid sequence alignment of the predicted Nedd2 protein with human (h) and mouse (m) ICE and, *C. elegans* CED-3 proteins is shown. Residues identical among all four proteins are indicated by an asterisk on top. The four Asp residues in the human ICE sequence at which the proteolytic self-cleavage is thought to occur (Thornberry et al. 1992) are encircled; (●) the possible cleavage sites in the Nedd2 protein. The corresponding locations in the Nedd2 protein of seven of the eight known missense mutations in *ced-3* that alter the amino acid residues (Yuan et al. 1993) are indicated by arrowheads. Two of these mutations involve the same Gly residue (Gly-321 in the Nedd2 protein) and are therefore indicated with a single arrowhead. (○) The active Cys residue of ICE, which is conserved in all four proteins.

sion roughly matches with the embryonic period when most of the neurogenesis occurs.

To determine whether *Nedd2* expression is limited to the cells of CNS lineage alone, we further analyzed *Nedd2* mRNA in sections of mouse embryos at E11.5 and E13.5 by in situ hybridization. Similar patterns were observed for both stages, and therefore only data for E13.5 are shown (Fig. 4A–H). *Nedd2* transcript was detected in most organs although the expression levels and spatial patterns were variable. High *Nedd2* expression was evident in the CNS (especially telencephalon and thalamus), liver, lung, kidney, and hair follicles of vibrissae (Fig. 4A). In the telencephalon, NPCs proliferate in the ventricular zone, migrate outward across the intermediate zone, and differentiate. *Nedd2* expression is higher in the ventricular zone than in the intermediate zone, the mantle zone, and the ependymal layer (Fig. 4C), which is similar to the distribution of Bcl-2 protein (Merry et al. 1994). Relatively strong signals are seen in developing glomeruli in renal cortex (Fig. 4D), where considerable cell death occurs during organogenesis (Coles et al. 1993). In the lung, bronchiolar epithelial cells expressing moderate levels of *Nedd2* mRNA surround shrunk cells with intense expression, which correlates with the ongoing cell death for canalization (Fig. 4E). In the liver, cell populations with moderate and weak expression of *Nedd2* form a reticular pattern (Fig. 4F), reminiscent of the lobular structure that occurs upon maturity. In the small intestine, *Nedd2* expression is high in the epithelial layer surrounding the lumen, moderate in serosa, and the lowest in mesenchyme (Fig. 4G). *Nedd2* is strongly expressed in hair follicles of vibrissae (Fig. 4H), where follicular cells are destined to die to form hair. Other tissues such as skin, oral mucosa,

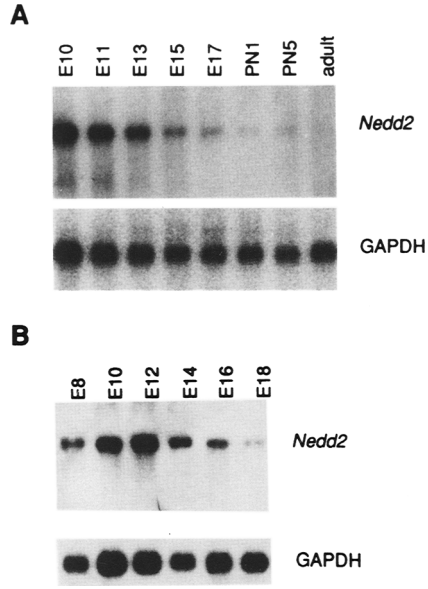

Figure 3. *Nedd2* mRNA expression is developmentally downregulated. (*A*) Northern analysis of poly(A)$^+$ RNA isolated from dissected heads (E10, E11, and E13) or brain (E15, E17, PN1, PN5, and adult) from embryonic (E) or postnatal (PN) animals. (*B*) Northern analysis of poly(A)$^+$ RNA isolated from total embryos at indicated postcoital days. At E8, RNA was isolated from embryos in utero. In both *A* and *B*, the blots were sequentially hybridized to a *Nedd2* probe and a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. The exposure time for *Nedd2*-hybridized blots was ~24 hr.

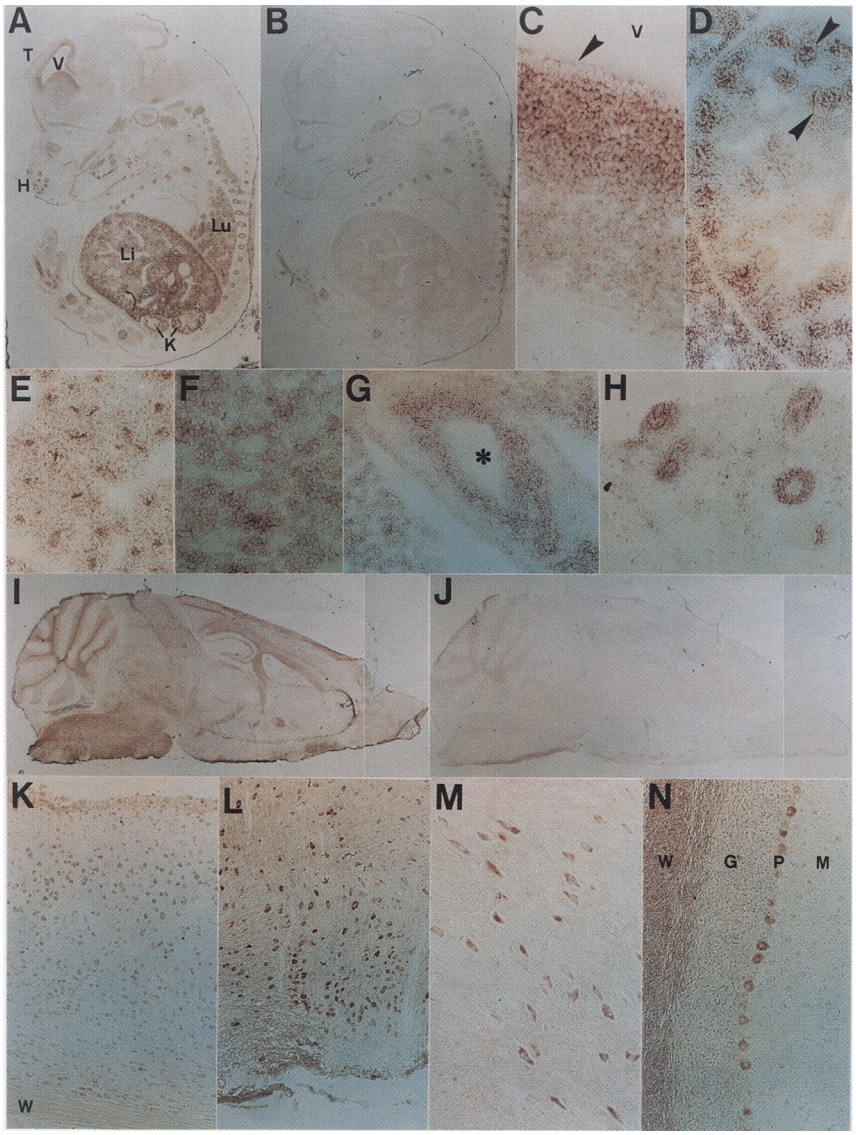

Figure 4. *Nedd2* mRNA expression in mouse embryos and adult brain. Bright-field photomicrographs of parasagittal sections of E13.5 hybridized with a *Nedd2* antisense probe (*A*) and a *Nedd2* sense probe (*B*). Note that the photographic exposure time for the sense control in *B* was approximately three times that for *A*. Positive signals (brown) are ubiquitously identified, but prominent in some organs. Higher magnification of telencephalon (*C*), kidney (*D*), lung (*E*), liver (*F*), small intestine (*G*), and hair follicles of vibrissae (*H*) show characteristic patterns of expression. Note the high expression of *Nedd2* in the ventricular zone between areas of lower expression, i.e., ependymal layer (arrowhead) and the mantle zone (*C*). Also note the developing glomeruli (arrowheads) in the renal cortex (*D*). A reticular pattern of expression is identified in the liver (*F*). Intestinal epithelia surrounding lumen (asterisk) show higher expression than outer layer epithelia (*G*). A parasagittal section of adult brain was hybridized with a *Nedd2* antisense probe (*I*) and a *Nedd2* sense probe (*J*). The photographic exposure time in *J* was twice as long as in *I*. Higher magnification of the cerebral cortex (*K*), nucleus gigantocellularis of the pons (*L*), nucleus ruber of the midbrain (*M*), and the cerebellar cortex (*N*) show variable degrees of expression in specific neurons. (T) telencephalon; (V) lateral ventricle; (Lu) lung; (Li) liver; (K) kidney; (H) hair follicles of vibrissae; (W) white matter; (G) granular layer; (P) Purkinje cell layer; (M) molecular layer.

skeletal muscle, submandibular gland, and thymus (data not shown) also show moderate levels of *Nedd2* transcript.

Nedd2 *is expressed in various adult tissues and cultured cells*

Although the expression of *Nedd2* mRNA is much higher in embryos, relatively low and varied levels of mRNA can be detected in all the adult tissues examined, including brain, spleen, heart, lung, testis, kidney, skeletal muscle, liver (Fig. 5A), thymus, ovary, and gut (data not shown). In situ analyses indicated that in adult brain *Nedd2* mRNA was present in most neurons, albeit at variable levels (Fig. 4I). Expression was seen clearly in the neurons of cerebral cortex, such as pyramidal cells (Fig. 4K), nucleus gigantocellularis of the pons (Fig. 4L), nucleus ruber of the midbrain (Fig. 4M), and Purkinje and granular cells in the cerebellum (Fig. 4N). All of the cultured cell lines including neuroblastoma (N18), glioma (C6), embryonal carcinoma (PCC4, P19, and F9), fibroblast (NIH-3T3), and pheochromocytoma (PC12) expressed varying levels of *Nedd2* mRNA (Fig. 5B). The highest expression was evident in N18 and PC12 cells (Fig. 5B).

Overexpression of Nedd2 *induces apoptosis in fibroblast and neuroblastoma cells*

To examine the biological effects of *Nedd2* expression, the complete coding region of the cDNA was cloned downstream of a chimeric cytomegalovirus immediate early (IE) enhancer/chicken β-actin promoter, in a eukaryotic expression vector pCXN2, carrying the *neo* gene as a selectable marker (Niwa et al. 1991) to generate pCXN2–N2. Expression vectors carrying the amino- and

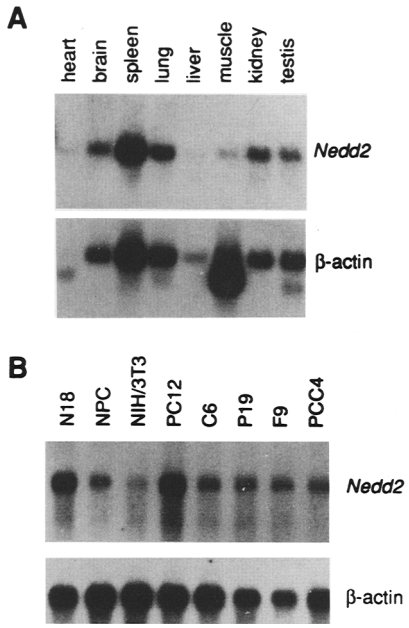

Figure 5. *Nedd2* mRNA is expressed in adult tissues and cultured cells. Northern analysis of poly(A)$^+$ RNA isolated from adult mouse tissues (*A*) and cultured cells (*B*). The blots were sequentially hybridized to a *Nedd2* probe and a human β-actin probe. The exposure time for *Nedd2*-hybridized blots was ~3 days.

carboxy-terminal coding regions corresponding to putative P20 (pCXN2–N2N) and P10 (pCXN2–N2C) subunits of ICE (Thornberry et al. 1992), a frameshift mutation of *Nedd2*-coding frame (pCXN2–N2M), and the amino-terminal region in the antisense orientation (pCXN2–N2AS) were also generated (Fig. 6A). Mouse fibroblast NIH-3T3 (Jainchill et al. 1969) and neuroblastoma N18 (Amano et al. 1971) cells were used to study the biological effects of *Nedd2*. While trying to establish stably transfected cells expressing mRNA for the introduced cDNAs, we noticed that both N18 and NIH-3T3 cells transfected with pCXN2–N2 gave rise to only 2–3% G418-resistant colonies as compared with cells transfected with vector control or other *Nedd2* constructs. Furthermore, these G418-selected cells did not express the transfected *Nedd2* mRNA, whereas the cells transfected with all other constructs expressed high levels of mRNA (5–10 times the endogenous level of *Nedd2* transcript; data not shown). This suggested to us that *Nedd2* overexpression has cytotoxic effects in these cells. This cytotoxicity was not likely due to the nonspecific effects of the *Nedd2* cDNA on *neo* gene activity because the frameshift mutation of cDNA (pCXN2-N2M), which differs from the wild type in four nucleotide residues only, had no cytotoxic effects (data not shown).

Both ICE and CED-3 have been shown to induce apoptosis in cultured cells (Miura et al. 1993). Therefore the cytotoxicity observed with the expression of *Nedd2* may also reflect cell death by apoptosis. Hence, in the following experiments, cells were transfected transiently and

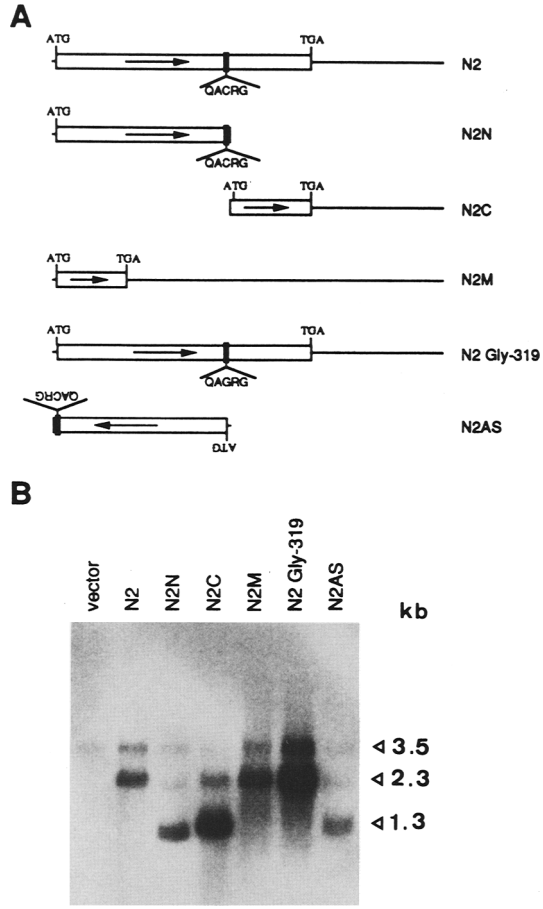

Figure 6. Structure of *Nedd2* constructs and their expression in transiently transfected N18 cells. (*A*) A diagrammatic representation of the various *Nedd2* cDNA constructs prepared for transfection studies. The ORF is shown as an open box; the noncoding region of cDNA is shown as a solid line. The locations of initiation and termination codons, and of the putative catalytic domain (QACRG), are indicated. In the N2N deletion mutant, the termination codon is provided by the vector. The N2AS mutant is similar to the N2N mutant except that the orientation in the expression vector, with respect to the promoter, is reversed. In N2 Gly-319, the wild-type Cys of N2 is replaced by a Gly residue. The orientation of each cDNA with respect to the β-actin promoter in the pCXN2 vector (*left* to *right*) is shown by an arrow. (*B*) Northern analysis of *Nedd2* expression in transiently transfected N18 cells. Approximately 4×10^5 cells on 60-mm dishes were transfected with the indicated cDNA construct in the pCXN2 vector and harvested 24 hr later. The entire amount of poly(A)$^+$ RNA recovered from the cells on each dish was subjected to Northern analysis. The transfected N2, N2M, and N2 Gly-319 constructs produce a transcript of ~2.3 kb, whereas N2N, N2AS, and N2C produce a transcript of ~1.3 kb. The additional 2.1-kb transcript seen in the N2N-, N2C-, and N2AS-transfected cells, most likely results from aberrant splicing and/or termination within the vector sequence. The intensity of the signals for the endogenous *Nedd2* transcript (3.5 kb) gives an approximate indication of the relative amount of RNA in each of the lanes. Essentially similar results were obtained with NIH-3T3 cells transfected with various constructs, although the signal intensities were three to four times weaker than for N18 cells, probably because of lower transfection efficiencies (see text).

examined for apoptosis. To determine the efficiency of transfection and to identify transfected cells, we cotransfected *Nedd2* expression plasmids with a β-galactosidase expression plasmid (pEF–βgal) by lipofection, stained the cells with X-gal 24 hr post-transfection, and examined positive blue cells morphologically for apoptosis. The estimated transfection efficiency for NIH-3T3 was 2–8%, and for N18 10–20%, in various experiments. As judged by Northern analyses, all of the transfected cDNAs were expressed efficiently (Fig. 6B). With the transfection efficiencies and cell death induced by the overexpression of pCXN2-N2 (see below) taken into account, the levels of mRNA transcribed from the pCXN2 constructs in these experiments were at least five times the endogenous transcript of *Nedd2* (Fig. 6B).

The most obvious observation from the cotransfection experiments was a 5- to 10-fold reduction in β-galactosidase-positive cells for the full-length *Nedd2* (pCXN2–N2) compared with vector control or other plasmid constructs, which produced a comparable number of blue transfected cells. This cytotoxic effect is consistent with the stable transfection experiments described above and indicates that the biological effects of *Nedd2* overexpression may be very strong and require only a short period after the expression vectors are introduced into the cells. Microscopic observations indicated that majority of the β-galactosidase-positive pCXN2–N2-transfected NIH-3T3 and N18 cells were smaller and condensed as opposed to the flat morphology of the cells transfected with other constructs (Fig. 7). Under higher magnification, fragmented cells and membrane-bound apoptotic bodies were evident for many of the blue cells (e.g., Fig. 7D,H). This effect on N18 and NIH-3T3 cells was only observed with the transfection of pCXN2–N2 carrying the complete coding region of *Nedd2* and not with the expression vector itself or expression construct carrying the amino-terminal (pCXN2–N2N) or carboxy-terminal (pCXN2–N2C) coding regions of the cDNA, or the amino-terminal coding region in the antisense orientation (pCXN2–N2AS) (Table 1). Furthermore, the frameshift mutant cDNA (pCXN2–N2M) also failed to show any biological effects on transfection into both cell lines (Table 1), indicating that the *Nedd2*-induced cell death is most likely attributable to the intact Nedd2 protein produced from cDNA and not some spurious consequence of the cDNA itself.

The Cys-285 in the QACRG pentapeptide of ICE is thought to be essential for its protease activity (Thornberry et al. 1992). To test whether the cell death caused by the overexpression of Nedd2 is also caused by its putative cysteine protease activity, we generated a substitution mutant of Nedd2 in which the Cys-319 of the conserved QACRG domain was replaced with a Gly (pCXN2–N2 Gly-319) (Fig. 6A). The overexpression of this mutated form of Nedd2 failed to induce apoptosis in both N18 and NIH-3T3 cells (Table 1), indicating that Cys-319 is essential for the biological activity of the Nedd2 protein.

Because of the relatively high transfection efficiency for N18 cells (10–20%), it was possible for us to analyze the DNA from the transiently transfected cells for fragmentation into nucleosomal repeats, a typical feature of apoptosis (Wyllie et al. 1980). Some fragmentation was evident in all samples, which we believe results from Lipofectamine treatment of these cells. However, the N18 cells transfected with pCXN2–N2 showed significantly stronger effects over the background (Fig. 8). These results substantiate further that *Nedd2* induces cell death by apoptosis.

Suppression of Nedd2*-induced apoptosis by* bcl-2

The *bcl-2* gene is known to protect certain mammalian cells from programmed cell death (Vaux et al. 1988; Nunez et al. 1990; Strasser et al. 1991; Sentman et al. 1991). *bcl-2* is also functionally similar to the *ced-9* gene in *C. elegans* (Vaux et al. 1992; Hengartner and Horvitz 1994), which probably functions by suppressing the activities of *ced-3* and *ced-4* genes (Yuan and Horvitz 1990). Moreover, *bcl-2* has been shown to inhibit apoptosis induced by both *ced-3* and ICE (Miura et al. 1993; Gagliardini et al. 1994). Because Nedd2 protein is structurally similar to CED-3 and ICE and induces apoptosis in mammalian cells (data shown above), it was interesting to test whether its biological activity could be suppressed by *bcl-2*. We generated NIH-3T3 and N18 cells constitutively expressing human *bcl-2* by transfecting a *bcl-2* expression vector. These cells were used for transient expression experiments with the *Nedd2* expression vectors used in the previous experiments. Approximately 60% of the β-galactosidase-positive NIH-3T3 and N18 cells transfected with the pCXN2–N2 now showed normal morphology, similar to the cells transfected with other expression constructs (Table 1; data not shown), suggesting that *bcl-2* can at least partially inhibit *Nedd2*-induced apoptosis in mammalian cells. This partial inhibition of *Nedd2*-induced apoptosis by *bcl-2* may reflect the clonal heterogeneity in the expression of *bcl-2*, because a pooled population of *bcl-2*-transfected cells was used in these experiments (see Materials and methods).

Chromosomal mapping of mouse Nedd2 *gene*

The mouse chromosomal location of *Nedd2* was determined by interspecific backcross analysis with progeny derived from matings of (C57BL/6J×*Mus spretus*)F_1 ×C57BL/6J mice. *Nedd2* is located in the proximal region of mouse chromosome 6 linked to *Ptn*, *Tcrb*, and *Hoxa*. Although 135 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 9), up to 193 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies by use of the additional data. The ratio of total number of mice exhibiting recom binant chromosomes to total number of mice analyzed for each pair of loci and the most likely gene order are centomere–*Ptn*-5/141–*Nedd2*-0/141–*Tcrb*-9/193–*Hoxa* (Fig. 9). The recombination frequencies [expressed as genetic distances in centiMorgans (cM)±the standard error] are pleiotrophin (Ptn)–3.6±1.6–[*Nedd2*, T-cell re-

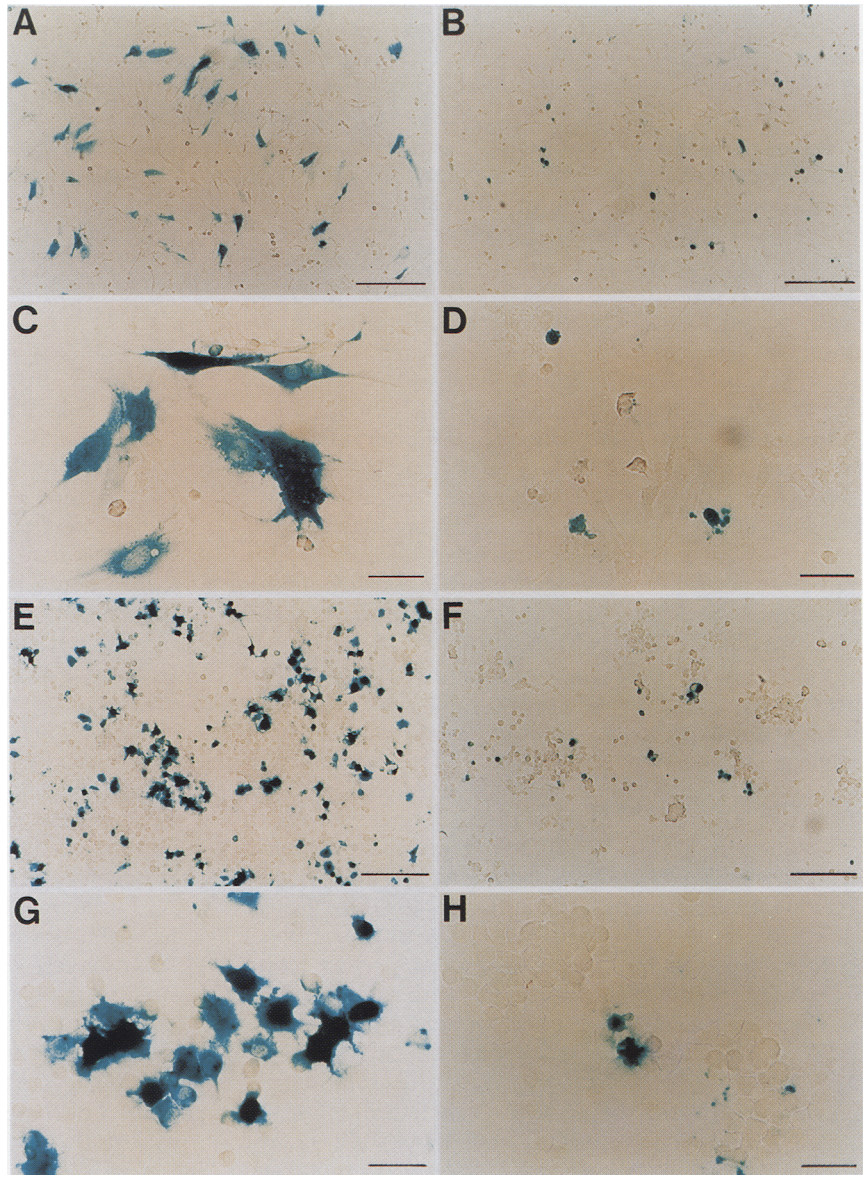

Figure 7. *Nedd2* induces cell death by apoptosis in NIH-3T3 and N18 cells. NIH-3T3 (*A–D*) or N18 (*E–H*) cells cotransfected with pEF–βgal and either pCXN2 vector (*A,C,E,G*) or pCXN2-N2 (*B,D,F,H*) were fixed and stained for 6 hr with X-gal. Morphology of typical blue cells is shown. A comparison of higher magnification observations of NIH-3T3 transfected with pCXN2 (*C*) or pCXN2-N2 (*D*) and N18 transfected with pCXN2 (*G*) or pCXN2-N2 (*H*) clearly indicates the apoptotic changes induced in *Nedd2* overexpressing cells. The NIH-3T3 and N18 cells transfected with pCXN2–N2N, pCXN2–N2AS, pCXN2–N2C, pCXN2–N2M, and pCXN2–N2 Gly-319 constructs show a morphology typical of pCXN2 vector-transfected cells (*A,C,E,G*) and are therefore not shown here. Bar in *A,B,E,F*, 200 μm; bar in *C,D,G*, and *H*, 40 μm.

ceptor β-chain (*Tcrb*)]–4.7±1.5–hoxa (*Hoxa*). No recombination was detected between *Nedd2* and *Tcrb* in 141 animals typed in common, suggesting that the two loci are within 2.1 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 6 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M.T. Davisson, T.H. Roderick, A.L. Hillyard, and D.P. Doolittle and provided from GBASE, a computerized data base maintained at The Jackson Laboratory, Bar Harbor, ME). *Nedd2* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).

Discussion

Previously, we had used a subtraction cloning approach to identify a set of novel mouse genes, *Nedd1–Nedd10*, which show developmentally down-regulated expression in the CNS (Kumar et al. 1992). As these genes are expressed at much higher levels in early embryonic CNS than in adult brain, they are likely to play some role in a development-related process, such as cell proliferation, differentiation, or PCD. In this study we have shown that *Nedd2* encodes a protein similar to the product of the *C. elegans* cell death gene *ced-3* and mammalian ICE, and induces apoptosis when overexpressed in cultured fibroblast and neuroblastoma cells. This suggests that *Nedd2* belongs to a family of mammalian genes in-

Table 1. *Overexpression of* Nedd2 *induces apoptosis in NIH-3T3 and N18 cells*

Expression construct	β-Galactosidase-positive apoptotic cells (%)			
	NIH-3T3	NIH/*bcl-2*	N18	N18/*bcl-2*
pCXN2 vector	1.6 ± 1.3	1.2 ± 0.6	2.4 ± 1.1	1.3 ± 0.2
pCXN2-N2	91.7 ± 5.6	34.0 ± 7.5	95.8 ± 3.9	29.3 ± 7.4
pCXN2-N2N	1.7 ± 1.3	N.D.	2.2 ± 1.2	1.7 ± 0.2
pCXN2-N2C	2.0 ± 1.5	N.D.	1.8 ± 0.6	1.7 ± 0.2
pCXN2-N2M	2.4 ± 2.3	N.D.	2.5 ± 0.7	2.2 ± 0.6
pCXN2-N2 Gly-319	1.8 ± 1.2	N.D.	1.9 ± 1.3	N.D.
pCXN2-N2AS	0.9 ± 0.9	0.7 ± 0.6	1.4 ± 1.1	1.2 ± 0.9

Various *Nedd2* expression constructs were cotransfected by lipofection with a β-galactosidase expression plasmid (pEF–βgal) into NIH-3T3, NIH-3T3 cells expressing human *bcl-2* (NIH/*bcl-2*), N18, and N18 cells expressing human *bcl-2* (N18/*bcl-2*). Cells were fixed 18–24 hr post-transfection and stained with X-gal for 6–24 hr, and numbers of blue cells with either normal flat or small round and apoptotic morphology were scored. The data (mean ± S.E.M.) are shown as percentage of morphologically apoptotic cells among all of the β-galactosidase positive blue cells. The data in the table were derived from observations on at least 2000 blue cells for each cell type transfected with various constructs and were collected from three to six independent experiments. (N.D.) Not determined.

cluding ICE, which, like *ced-3* in *C. elegans*, function in the regulation of PCD.

ICE is a cysteine protease that converts the inactive 31-kD pro-IL-1β to 17.5-kD active polypeptide by cleaving the precursor between Asp-116 and Ala-117 (Cerretti et al. 1992; Thornberry et al. 1992). ICE itself undergoes self-cleavage to generate two subunits, P20 and P10, both of which are required for the enzyme activity of the mature protein (Thornberry et al. 1992). The Cys-285 of ICE is essential for its catalytic activity (Thornberry et al. 1992). A comparison of protein sequences of Nedd2, ICE, and CED-3 indicates that the most homologous region among these proteins encompasses the P20 and P10 regions of ICE (Fig. 2), suggesting that this may be the region required for the biological activity of these proteins. Miura et al. (1993) have convincingly demonstrated that ICE cDNA corresponding to P20 + P10 alone can induce apoptosis in Rat-1 cells. As in ICE and CED-3 (Yuan et al. 1993), the pentapeptide QACRG, containing the active Cys residue, is also completely conserved in the Nedd2 protein. Furthermore, a substitution of this Cys residue by Gly abolished the apoptosis-inducing activity of Nedd2, suggesting that Nedd2 also functions as a cysteine protease. Interestingly, the amino acid residues corresponding to the seven of the eight known missense mutations of CED-3 (Yuan et al. 1993) are conserved in Nedd2 protein and therefore may be important for its biological activity.

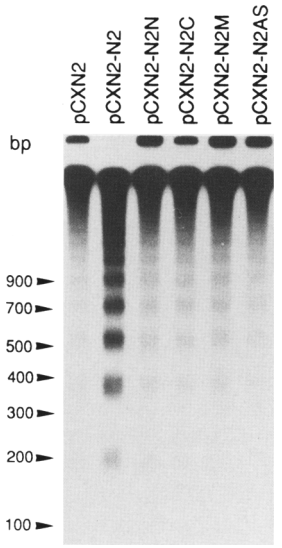

Figure 8. DNA fragmentation in *Nedd2*-transfected N18 cells. Genomic DNA isolated from N18 cells transfected with the indicated plasmid constructs were labeled with [α-^{32}P]dCTP and analyzed on 1.8% agarose gel as described by Rosl (1992). The positions of the markers (100-bp ladder from Life Technologies) are shown at *left*.

Overexpression of both ICE and *ced-3* has been shown to induce apoptosis in cultured Rat-1 cells (Miura et al. 1993). In addition, ICE also induces cell death in chicken dorsal root ganglia neurons, and this cell death can be prevented by the cowpox virus *crmA* gene (Gagliardini et al. 1994), a specific inhibitor of ICE (Ray et al. 1992). The results presented in this study show that overexpression of *Nedd2* gene results in apoptosis in mouse fibroblast and neuroblastoma cell lines. Only the full-length coding region of the *Nedd2* cDNA was active in inducing apoptosis. Expression of either the amino-terminal 327 amino acids corresponding to unprocessed P20 subunit, or the carboxy-terminal 99 amino acids corresponding to the P10 subunit of ICE alone, was insufficient to induce apoptosis, indicating that both corresponding subunits of Nedd2 protein may be required for its biological activity, as has been shown for ICE (Miura et al. 1993). The cleavage of the ICE precursor to produce P10 and P20 subunits has been shown to require active ICE (Thornberry et al. 1992). The in vitro translation of *Nedd2* mRNA results in a major product of 51 kD and some minor polypeptides. Incubation of this 51-kD Nedd2 protein at ambient temperature for up to 24 hr showed no detectable cleavage into smaller peptides nor there was an increase in the intensity of the smaller bands detected in the translation

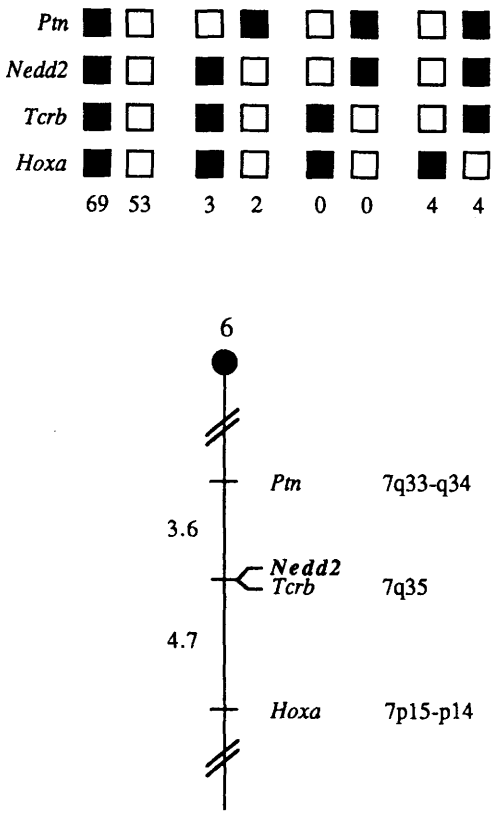

Figure 9. *Nedd2* maps in the proximal region of mouse chromosome 6. *Nedd2* was placed on mouse chromosome 6 by interspecific backcross analysis. The segregation patterns of *Nedd2* and flanking genes in 135 backcross animals that were typed for all loci are shown at the *top*. For individual pairs of loci, >135 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J×*M. spretus*) F_1 parent. (■) The presence of a C57BL/6J allele; (□) the presence of *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 6 linkage map with the location of *Nedd2* in relation to linked genes is shown at the *bottom*. Recombination distances between loci in centiMorgans are shown to the *left* of the map, and the positions of the loci in human chromosomes, where known, are shown to the *right*. References for the human map positions of loci cited in this study can be obtained from Genome Data Base maintained by The William H. Welch Medical Library of The John Hopkins University (Baltimore, MD).

reaction (data not shown). Whether Nedd2 protein is also cleaved into P20- and P10-like subunits of ICE and whether this cleavage requires the presence of active Nedd2 protein remains to be determined. The apoptosis induced by *Nedd2* was inhibited by *bcl-2* expression, suggesting that these two genes play opposing roles in cell death, an observation consistent with the data for both *ced-3* and ICE (Miura et al. 1993). As suggested for ICE (Miura et al. 1993), Nedd2 may also function by either activating some protein(s) required for cell death or inactivating protein(s) necessary for cell survival. A block in *bcl-2* function may be required for *Nedd2* to induce cell death.

We have shown that during embryonic development, *Nedd2* is expressed at relatively high levels in several tissues, including the CNS, liver, lung, and kidney (Fig. 4). Both CNS and kidney development is known to be associated with massive cell death (Oppenheim 1991; Coles et al. 1993), and a similar situation might exist in other tissues such as lung and liver. High expression of *Nedd2* in these tissues may therefore correlate with high PCD. Interestingly the expression pattern of *Nedd2* mRNA in developing CNS resembles that of Bcl-2 protein (Merry et al. 1994). *Nedd2* is also expressed in various adult tissues and cell lines of different origins. We also show that most of the postmitotic neurons of adult brain express *Nedd2* mRNA, some of them at moderate levels (Fig. 4). This raises an interesting question about the possible role of *Nedd2* in postmitotic cells. Both our studies with *Nedd2* and previous studies with ICE (Miura et al. 1993; Gagliardini et al. 1994) demonstrate that overexpression of these proteins in mammalian cells leads to apoptosis. From the expression data of cultured cells, such as growing neuroblastoma N18 and pheochromocytoma PC12 (Fig. 5B) cells, it is clear that moderate levels of *Nedd2* have no obvious effects on the growth and survival of these cells. Therefore, the physiological relevance of these overexpression experimental systems remains to be established. Factors such as those that regulate the processing of the inactive precursor to active protein, the presence of target protein(s) for these putative cysteine proteases, and the concentration of proteins that block cell death, like members of the Bcl-2 family (for review, see Williams and Smith 1993), may be crucial under physiological conditions. Postmitotic neurons and other cell types expressing moderate levels of Nedd2 may have other proteins to protect them from Nedd2-induced PCD. The levels of Bcl-2 protein are substantially reduced in the nervous system with aging (Merry et al. 1994); therefore, a balance between Nedd2 and Bcl-2 proteins may be important in neuronal cell death associated with aging and in human disorders characterized by neuronal degeneration.

The known substrate for ICE, IL-1β, is expressed by a limited number of cell types such as peripheral blood monocytes (Cerretti et al. 1992). On the other hand, ICE expression has been detected in a variety of tissues (Cerretti et al. 1992; Miura et al. 1993), suggesting that ICE may have additional substrates. *Nedd2* is also expressed in various tissues and induces apoptosis in at least two different cell types used in our study, indicating a possible functional redundancy between ICE and *Nedd2*. It is possible, however, that Nedd2 and ICE act on different but functionally related substrates and that there are still other Nedd2/ICE-like proteins in the mammalian cells providing additional complexity to the cell death machinery.

Oncogenes such as *bcl-2* act by enhancing cell viability (for review, see Korsmeyer 1992), whereas a tumor suppressor gene such as *p53* may be required for normal physiological cell death to occur (Yonish-Rouach et al.

1991; Shaw et al. 1992). Therefore it is likely that genes like *Nedd2* and ICE, which induce apoptosis, may also function as tumor suppressor genes and may be important in multistep carcinogenesis. The mouse chromosomal mapping of *Nedd2* localizes it to the proximal region of chromosome 6, near T-cell receptor β-chain gene (Fig. 9). This region of mouse chromosome 6 shares a region of homology with human chromosome 7q. Structural abnormalities involving chromosome 7q occur frequently in a number of human hematological neoplasms (Johansson et al. 1993); therefore, it would be worthwhile to map *Nedd2* in humans and to check whether *Nedd2* is associated with any human disorders, including malignancies and/or neurological syndromes.

Materials and methods

Cell culture

NPCs were isolated and cultured as described before (Kitani et al. 1991). Embryonal carcinoma (EC) cell lines, P19 (Edwards and McBurney 1983) and PCC4 (Jakob et al. 1973), were maintained in Eagle's minimum essential medium with α modification (α-MEM) supplemented with 10% fetal bovine serum (FCS). The mouse fibroblast cell line NIH-3T3 (Jainchill et al. 1969) and the mouse neuroblastoma cell line N18 (Amano et al. 1971) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. All other cell lines used for RNA extraction were maintained as described previously (Kumar et al. 1994).

cDNA cloning

The original *Nedd2* clone 2C8, obtained from the subtracted NPC cDNA library-contained a cDNA insert of 0.8 kb from the 3′ end of the mRNA (Kumar et al. 1992). By use of this insert we rescreened the mouse NPC cDNA library (Kumar et al. 1992) to obtain a longer cDNA clone (2C8.8) of 1.2 kb, which was used in further screening of a PCC4 cell-line cDNA library (Stratagene). Two overlapping clones of 2.6 kb (PC 2C8.1) and 2.3 kb (PC 2C8.2), isolated covering a 3.7-kb DNA, were sequenced in their entirety (data base entry D10713). This sequence contained a short ORF of 171 amino acids preceded by a long noncoding sequence. We therefore suspected that the 5′ region of the clone may have arisen from cloning artifacts and isolated additional cDNA clones from mouse spleen and lung cDNA libraries (both from Stratagene). Among several positive clones isolated, four spleen clones (MS N2.2, 3, 4, and 5) and a lung clone (ML N2.2) with overlapping maps and no apparent fusion with other cDNAs were further characterized. The sequence derived from these clones indicated that ~1.8 kb of 5′ sequence of the clone PC 2C8.1 had originated from some cloning artifact. Approximately 500,000 plaques from each library were screened by use of standard protocols (Sambrook et al. 1989). After three cycles of plaque purification, the pBluescript SK(−) from these clones was rescued according to the instructions supplied by the manufacturer (Stratagene), and both strands were sequenced from exonuclease III-generated nested deletions and sequence-specific primers by use of a Sequenase DNA sequencing kit (U.S. Biochemical).

Computer analyses of the nucleotide and protein sequences were performed by use of the Wisconsin Genetic Computer Group program package. Data base sequence searches were performed by FASTA and TFASTA (Pearson and Lipman 1988). Sequence alignment and homology computations were also performed at the National Center for Biotechnology Information (NCBI) by use of the BLAST network service. For in vitro transcription from *Nedd2* cDNA clones, MS N2.4, 5, and 6 were linearized with either *Bam*HI or *Xho*I, and capped mRNA was synthesized with a kit (Stratagene). Translation of mRNA was performed by the *In Vitro* Express kit according to the instructions supplied by the manufacturer (Stratagene) and analyzed on 12.5% polyacrylamide gels (Sambrook et al. 1989).

RNA isolation and analysis

Poly(A)$^+$ RNA was isolated from quick-frozen tissues or cultured cells by use of the Fast Track kit (Invitrogen) or by oligo(dT) batch absorption (Sambrook et al. 1989). Approximately 2.5 μg of the poly(A)$^+$ RNA samples was electrophoresed on 1.2% agarose/2.2 M formaldehyde gels, transferred to Biodyne A membrane (Pall), and hybridized to probes labeled with [α-^{32}P]dCTP (Amersham) by random priming with the Klenow fragment of DNA polymerase (Sambrook et al. 1989). In Figure 5B, a commercially obtained mouse multiple tissue poly(A)$^+$ RNA blot (Clonetech) was used. Entire cDNA inserts derived from clone 2C8.8 (Figs. 3 and 5) and MS N2.4 (Fig. 6) were used as probes for *Nedd2*. The human β-actin and glyceraldehyde 3-phosphate dehydrogenase probes were from Clontech.

In situ hybridization

The procedure was adapted from Chun et al. (1991) and digoxigenin (DIG) labeling and detection system protocols (Boehringer Mannheim). In brief, embryos at various developmental stages or dissected brain from adult animals were cryostat-sectioned at a thickness of 10 μm and mounted on glass slides treated with Vectabond (Vector Laboratories). After pretreatments, sections were hybridized with DIG-labeled sense and antisense RNA probes in a buffer (50% formamide, 2× SSPE, 10 mM dithiothreitol, 2 mg/ml of yeast tRNA, 0.25 mg/ml of bovine serum albumin, 0.5 mg/ml of denatured salmon sperm DNA, and 0.5 mg/ml of polyadenylic acid) at 42°C for 18 hr. The DIG-labeled RNA probes were transcribed from the *Xba*I-linearized (for sense) or *Hin*dIII-linearized (for antisense) plasmid PC 2C8.2 according to the Boehringer Mannheim protocol and limit-alkali-digested to 100–200 bases. Several different concentrations of DIG-labeled RNA probes were used in the hybridization experiments with essentially similar results. After hybridization, the sections were treated with 40 μg/ml of RNase A at 37°C for 15–30 min, washed in 0.2× SSPE at 42°C, and color-developed following the manufacturer's protocol (Boehringer). Further confirmation of the specificity of the *Nedd2* signals was obtained by the use of [^{35}S]-labeled UTP-labeled probes according to published protocols (Sazuka et al. 1992a), which produced results similar to those obtained with DIG-labeled probes (data not shown).

Construction of expression vectors

Nedd2 expression plasmids were constructed in the pCXN2 (Niwa et al. 1991) vector background. To construct the full-length *Nedd2* expression plasmid (pCXN2–N2), the cDNA released from clone MS N2.4 by *Bam*HI–*Xho*I digestion was blunt-ended with T4 DNA polymerase and cloned into the *Eco*RI-digested and T4 DNA polymerase-treated vector downstream of chicken β-actin promoter (Niwa et al. 1991). To construct vectors expressing amino-terminal (pCXN2–N2N) and carboxy-terminal (pCXN2–N2C) regions of the Nedd2 protein, MS N2.4 was digested with *Sal*I–*Bam*HI, and the resulting gel-

purified fragments of ~1.0 kb (amino-terminal) and 1.1 kb (carboxy-terminal) treated with T4 DNA polymerase and cloned into blunt-ended pCXN2 as described above. pCXN2–N2N contains the coding sequence for the amino-terminal 327 amino acid residues, starting at the first-inframe Met (Fig. 1B). pCXN2–N2C is likely to encode either a 99- or 97-amino-acid carboxy-terminal polypeptide, depending on whether translation starts at Met-353 or Met-355 (residue numbers in intact Nedd1 protein) (Fig. 1B). An antisense expression vector (pCXN2–N2AS) was constructed by cloning the blunt-ended 1.0-kb *Sal*I fragment into the antisense orientation with respect to the β-actin promoter. A frameshift mutation (pCXN2–N2M) was created by *Sac*I digestion/T4 DNA polymerase treatment and self-ligation of *Nedd2* cDNA in the pCXN2 vector. This results in the removal of four nucleotides (221–224 in Fig. 1B), thereby shifting the frame, which now terminates at nucleotide 398 (Fig. 1B) and potentially encodes a protein of 129 amino acid residues. Site-directed mutagenesis to substitute the T residue at 961 (Fig. 1B) with a G residue (Cys → Gly) was carried out according to the protocol of Kunkel et al. (1987), with single-stranded DNA from clone MS N2.4 as a template and a 22-mer oligonucleotide corresponding to nucleotides 950–971 (Fig. 1B) and carrying the substitution as a primer. The mutated cDNA insert was released by *Bam*HI–*Xho*I digestion and cloned into pCXN2 as described above, to generate pCXN2–N2 Gly-319. To check whether all constructs produced proteins of desired sizes, where necessary, cDNA fragments were cloned into pBluescript, and mRNA was generated from either *Bam*HI- or *Xho*I-linearized templates by in vitro transcription as described above. Aliquots of mRNA were translated with *In Vitro* Express extracts (Stratagene) and analyzed on 12.5% polyacrylamide gels as described above. The approximate sizes of the protein products for N2, N2N, N2C, N2M, and N2 Gly-319 were 51, 36, 11, 15, and 51 kD, respectively, whereas no product was detected for N2AS (data not shown).

Cell transfection

Cells were plated at a density of 2×10^5 cells/35-mm dish or 5×10^5 cells/60-mm dish. The following day, 2.5 or 7.5 μg of the pCXN2 plasmid constructs and, where indicated, 0.5 or 1.5 μg of a β-galactosidase expression vector (pEF-βgal) were cotransfected with 10 or 30 μl of Lipofectamine (Life Technologies). pEF–βgal was kindly provided by K. Kataoka and contains the *E. coli lacZ* gene in a pEF–BOS (Mizushima and Nagata 1990) background. For β-galactosidase expression analysis, at 24 hr post-transfection cells were rinsed with PBS, fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, washed twice with PBS, and stained for 6–24 hr with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. After rinsing in PBS, positive cells were microscopically observed for apoptosis and photographed. To produce *bcl-2*-expressing cells, N18 and NIH-3T3 cells were stably transfected with a expression vector carrying human *bcl-2* under control of Rous sarcoma virus (RSV) promoter and hygromycin as a selection marker (kindly provided by David Vaux) by lipofection (Life Technologies). Transfected cells were selected with hygromycin for 2 weeks and pooled. Expression of Bcl-2 protein was analyzed by immunoblots using a commercial antibody (DAKO) (data not shown).

Genomic DNA extraction and electrophoresis

Cells transfected by use of Lipofectamine were scraped into medium 24 hr post-transfection and collected by centrifugation. Genomic DNA was isolated by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation (Sambrook et al. 1989). DNA (0.5–1 μg) was labeled with [α-^{32}P]dCTP with the Klenow fragment of DNA polymerase and analyzed on 1.8% agarose gel for DNA fragmentation as described by Rosl (1992).

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J×*M. spretus*) F_1 females and C57BL/6J males as described (Copeland and Jenkins 1991). A total of 205 N_2 mice were used to map the *Nedd2* locus. Southern hybridizations were performed as described previously (Jenkins et al. 1982). The *Nedd2* probe, a 2.0-kb *Eco*RI fragment of PC 2C8.2 representing the 3′ end of the cDNA, detects a 3.9- or 4.5-kb band in *Xba*I-digested C57BL/6J and *M. spretus* DNA, respectively. The presence or absence of the 4.5-kb *M. spretus*-specific *Xba*I fragment was followed in backcross mice. A description of the probes and RFLPs for the loci linked to *Nedd2* including *Ptn*, *Tcrb*, and *Hoxa* (formerly *Hox-1*) has been reported previously (Siracusa et al. 1991; Li et al. 1992). Recombination distances were calculated as described (Green 1981) with a computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Accession number

The sequence reported in this paper will appear in GenBank, DDBJ, EMBL, and NCBI data bases under accession number D28492. Readers should note that the gene symbol *Nedd2* used in this paper has been approved by the International Committee on Mouse Genetic Nomenclature and replaces *NEDD-2*, used by us previously (Kumar et al. 1992), or *nedd-2*, used by others (Yuan et al. 1993; Vaux et al. 1994) to define the same locus.

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References

Amano, T., E. Richelson, and M. Nirenberg. 1971. Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci.* **69:** 258–263.

Cerretti, D.P., C.J. Kozlosky, B. Mosley, N. Nelson, K.V. Ness, T.A. Greenstreet, C.J. March, S.R. Kronheim, T. Druck, L.A. Cannizzaro, K. Huebner, and R.A. Black. 1992. Molecular cloning of the interleukin-1β converting enzyme. *Science* **256:** 97–100.

Chun, J.J.M., D.G. Schatz, M.A. Oettinger, R. Jaenisch, and D. Baltimore. 1991. The recombination activating gene-1

(RAG-1) transcript is present in the murine central nervous system. *Cell* **64:** 189–200.

Cleary, M.L., S.D. Smith, and J. Sklar. 1986. Cloning and structural analysis of cDNAs for *bcl-2* and a hybrid *bcl-2*/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47:** 19–28.

Coles, H.S.R., J.F. Burne, and M.C. Raff. 1993. Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* **118:** 777–784.

Copeland, N.G. and N.A. Jenkins. 1991. Development and applications of a molecular genetic linkage map of the mouse genome. *Trends Genet.* **7:** 113–118.

Cowan, W.M., J.W. Fawcett, D.D.M. O'Leary, and B.B. Stanfield. 1984. Regressive events in neurogenesis. *Science* **225:** 1258–1265.

Davies, A.M. 1987. Molecular and cellular aspects of patterning sensory neuron connections in the vertebrate nervous system. *Development* **101:** 185–208.

Edwards, M.K.S. and M.W. McBurney. 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev. Biol.* **98:** 187–191.

Ellis, H.M. and H.R. Horvitz. 1986. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44:** 817–829.

Ellis, R.E., J. Yuan, and H.R. Horvitz. 1991. Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7:** 663–698.

Gagliardini, V., P.-A. Fernandez, R.K.K. Lee, H.C.A. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. Prevention of vertebrate neuronal death by *crmA* gene. *Science* **263:** 826–828.

Glucksmann, A. 1951. Cell death in normal vetebrate ontogeny. *Biol. Rev.* **26:** 59–86.

Green, E.L. 1981. Linkage, recombination and mapping. In *Genetics and probability in animal breeding experiments*, pp. 77–113. Oxford University Press, New York.

Hengartner, M.O. and H.R. Horvitz. 1994. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. *Cell* **76:** 665–676.

Hengartner, M.O., R.E. Ellis, and H.R. Horvitz. 1992. *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death. *Nature* **356:** 494–499.

Jainchill, J.L., S. Aaronson, and G. Todaro. 1969. Murine sarcoma and leukemia viruses: Assay using clonal lines of contact-inhibited mouse cells. *J. Virol.* **4:** 549–553.

Jakob, H., T. Boon, J. Gaillard, J.-F. Nicolas, and F. Jacob. 1973. Teratocarcinome de la spuris: Isolement culture et proprietes de cellules a potentialites multiples. *Ann. Microbiol. (Inst. Pasteur)* **124B:** 269–282.

Jenkins, N.A., N.G. Copeland, B.A. Taylor, and B.K. Lee. 1982. Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. *J. Virol.* **43:** 26–36.

Johansson, B., F. Mertens, and F. Mitelman. 1993. Cytogenetic deletion map of hematologic neoplasms: Circumstantial evidence for tumor suppressor loci. *Genes Chromosomes Cancer* **8:** 205–218.

Kerr, J.F.R., A.H. Wyllie, and A.R. Currie. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26:** 239–257.

Kitani, H., R. Shiurba, T. Sakakura, and Y. Tomooka. 1991. Isolation and characterization of mouse neural precursor cells in primary culture. *In vitro Cell. Dev. Biol.* **27A:** 615–624.

Korsmeyer, S.J. 1992. *Bcl-2* initiates a new category of oncogenes: Regulators of cell death. *Blood* **80:** 879–886.

Kozak, M. 1986. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15:** 8125–8132.

Kumar, S., Y. Tomooka, and M. Noda. 1992. Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem. Biophys. Res. Commun.* **185:** 1155–1161.

Kumar, S., M. Iwao, T. Yamagishi, M. Noda, and M. Asashima. 1993a. Expression of GTP-binding protein gene *drg* during *Xenopus laevis* development. *Int. J. Dev. Biol.* **37:** 539–546.

Kumar, S., Y. Yoshida, and M. Noda. 1993b. Cloning of a cDNA which encodes a novel ubiquitin-like protein. *Biochem. Biophys. Res. Commun.* **195:** 393–399.

Kumar, S., T. Matsuzaki, Y. Yoshida, and M. Noda. 1994. Molecular cloning and biological activity of a novel developmentally regulated gene encoding a protein with β-transducin-like structure. *J. Biol. Chem.* **269:** 11318–11326.

Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154:** 367–382.

Li, Y.-S., R.M. Hoffman, M.M. LeBeau, R. Espinosa III, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, and T.F. Deuel. 1992. Characterization of the human pleiotrophin gene. *J. Biol. Chem.* **267:** 26011–26016.

Merry, D.E., D.J. Veis, W.F. Hickey, and S.J. Korsmeyer. 1994. bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* **120:** 301–311.

Miura, M., H. Zhu, R. Rotello, E.A. Hartwieg, and J. Yuan. 1993. Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammlian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75:** 653–660.

Mizushima, S. and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18:** 5322.

Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection of high-expression transfectants with a novel eukaryotic vector. *Gene* **108:** 193–200.

Nunez, G., L. London, D. Hockenbery, M. Alexander, J.P. McKearn, and S.J. Korsmeyer. 1990. Deregulated *bcl-2* gene expression selectively prolongs survival of growth-factor deprived hematopoietic cell lines. *J. Immunol.* **144:** 3602–3610.

Oppenheim, R.W. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14:** 453–501.

Pearson, W.R. and D.J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci.* **85:** 2444–2448.

Raff, M.C. 1992. Social controls on cell survival and cell death. *Nature* **356:** 397–400.

Ray, C.A., R.A. Black, S.R. Kronheim, T.A. Greenstreet, P.R. Sleath, G.S. Salvesen, and D.J. Pickup. 1992. Viral inhibition of inflammation: Cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell* **69:** 597–604.

Rosl, F. 1992. A simple method for detection of apoptosis in human cells. *Nucleic Acids Res.* **20:** 5243.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sazuka, T., M. Kinoshita, Y. Tomooka, Y. Ikawa, M. Noda, and S. Kumar. 1992a. Expression of DRG during murine embryonic development. *Biochem. Biophys. Res. Commun.* **189:** 371–377.

Sazuka, T., Y. Tomooka, Y. Ikawa, M. Noda, and S. Kumar. 1992b. DRG: A novel developmentally regulated GTP-binding protein. *Biochem. Biophys. Res. Commun.* **189:** 363–370.

Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. *bcl-2* inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* **67:** 879–888.

Shaw, P., R. Bovey, S. Tardy, R. Sahli, B. Sordat, and J. Costa. 1992. Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci.* **89:** 4495–4499.

Siracusa, L.D., M.H. Rosner, M.A. Vigano, D.J. Gilbert, L.M. Staudt, N.G. Copeland, and N.A. Jenkins. 1991. Chromosomal location of the octamer transcription factors, *Otf-1*, *Otf-2*, and *Otf-3*, defines multiple *Otf-3*-related sequences dispersed in the mouse genome. *Genomics* **10:** 313–326.

Strasser, A., A.W. Harris, and S. Cory. 1991. *bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67:** 889–899.

Sulston, J.E. and H.R. Horvitz. 1977. Post embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82:** 110–156.

Sulston, J.E., E. Schierenberg, J.G. White, and N. Thomson. 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100:** 64–119.

Thornberry, N.A., H.G. Bull, J.R. Calaycay, K.T. Chapman, A.D. Howard, M.J. Kostura, D.K. Miller, S.M. Molineaux, J.R. Weidner, J. Aunins, K.O. Elliston, J.M. Ayala, F.J. Casano, J. Chin, G.J.-F. Ding, L.A. Egger, E.P. Gaffney, G. Limjuco, O.C. Palyha, S.M. Raju, A.M. Rolando, J.P. Salley, T.-T. Yamin, T.D. Lee, J.E. Shively, M. MacCross, R.A. Mumford, J.A. Schmidt, and M.J. Tocci. 1992. A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* **356:** 768–774.

Tsujimoto, Y. and C.M. Croce. 1986. Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci.* **83:** 5214–5218.

Vaux, D.L., S. Cory, and J.M. Adams. 1988. *bcl-2* gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. *Nature* **335:** 440–442.

Vaux, D.L., I.L. Weissman, and S.K. Kim. 1992. Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. *Science* **258:** 1955–1957.

Vaux, D.L., G. Haecker, and A. Strasser. 1994. An evolutionary perspective on apoptosis. *Cell* **76:** 777–779.

Williams, G.T. and C.A. Smith. 1993. Molecular regulation of apoptosis: Genetic controls on cell death. *Cell* **74:** 777–779.

Wyllie, A.H., J.F.R. Kerr, and A.R. Currie. 1980. Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68:** 251–306.

Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type p53 induces apoptosis of myeloid leukaemia cells that is inhibited by interleukin-6. *Nature* **352:** 345–347.

Yuan, J. and H.R. Horvitz. 1990. Genetic mosaic analyses of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. *Dev. Biol.* **138:** 33–41.

Yuan, J. and H.R. Horvitz. 1992. The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116:** 309–320.

Yuan, J., S. Shaham, S. Ledoux, H. Ellis, and H.R. Horvitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell* **75:** 641–652.

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