Physiol Genomics 30: 156–171, 2007.
First published March 20, 2007; doi:10.1152/physiolgenomics.00249.2006.

Single *mage* gene in the chicken genome encodes CMage, a protein with functional similarities to mammalian type II Mage proteins

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Submitted 12 November 2006; accepted in final form 20 March 2007

López-Sánchez N, González-Fernández Z, Niinobe M, Yoshikawa K, Frade JM. Single *mage* gene in the chicken genome encodes CMage, a protein with functional similarities to mammalian type II Mage proteins. *Physiol Genomics* 30: 156–171, 2007. First published March 20, 2007; doi:10.1152/physiolgenomics.00249.2006.—In mammals, the type II melanoma antigen (Mage) protein family is constituted by at least 10 closely related members that are expressed in different tissues, including the nervous system. These proteins are believed to regulate cell cycle withdrawal, neuronal differentiation, and apoptosis. However, the analysis of their specific function has been complicated by functional redundancy. In accordance with previous studies in teleosts and *Drosophila*, we present evidence that only one *mage* gene exists in genomes from protists, fungi, plants, nematodes, insects, and nonmammalian vertebrates. We have identified the chicken *mage* gene and cloned the cDNA encoding the chick Mage protein (CMage). CMage shares close homology with the type II Mage protein family, and, as previously shown for the type II Mage proteins Necdin and Mage-G1, it can interact with the transcription factor E2F-1. CMage is expressed in specific regions of the developing nervous system including the retinal ganglion cell layer, the ventral horn of the spinal cord, and the dorsal root ganglia, coinciding with the expression of the neurotrophin receptor p75 ($p75^{NTR}$) in these regions. We show that the intracellular domain of $p75^{NTR}$ can interact with both CMage and Necdin, thus preventing the binding of the latter proteins to the transcription factor E2F-1, and facilitating the proapoptotic activity of E2F-1 in N1E-115 differentiating neurons. The presence of a single *mage* gene in the chicken genome, together with the close functional resemblance between CMage and Necdin, makes this species ideal to further analyze signal transduction through type II Mage proteins.

Necdin; $p75^{NTR}$; E2F-1

THE MELANOMA ANTIGEN (Mage) proteins were initially described as precursors of human antigens exposed by the members of the major histocompatibility complex in melanoma cells. This protein family has since been characterized by the presence of a 165- to 171-amino acid Mage homology domain (MHD) in the center of the molecule (5). The first member of this protein superfamily to be identified was Mage-A1 (59). However, the gene encoding Mage-A1 was later found to belong to a cluster of 15 Mage-A genes located in the q28 region of the human X chromosome (10, 15). Subsequent studies identified further groups of related *mage* genes in two clusters on the human X chromosome. These genes encode 17 Mage-B proteins (10, 39, 41), and seven Mage-C proteins (10, 39, 40). Similarly, two groups of *mage* genes of murine origin have also been identified to date (13, 14, 48). Based on sequence homology, these two groups were considered as the murine counterparts of the human *mage-A* and *mage-B* genes. Accordingly, the *mage-C* genes do not seem to be present in the mouse genome, and their absence suggests that the members of this subfamily have arisen during the course of mammalian evolution. A common feature of all the mammalian genes encoding the Mage-A, -B, and -C proteins is that their open reading frames are contained within a single exon and that their normal expression in adults is restricted to male germinal cells and placenta. These features together with their genomic clustering have led to their classification as type I Mage proteins (5).

At least 10 new human genes encoding proteins that contain a Mage domain and that reside outside of the *mage*-A, -B, and -C clusters have been described so far. As a result, the Mage protein superfamily has recently been expanded (10) to include this type II family of Mage proteins, comprising: Mage-D1/NRAGE/Dlxin-1, Mage-D2, Mage-D3/Trophinin/Magphinin, Mage-E1/Mage-D4, Mage-E2, Mage-F1 Mage-G1/Necdin-like 2, Mage-H1, Mage-L2, and Necdin (5). These proteins contain a phylogenetically distinct MHD, although like type I Mage proteins, most of them are encoded by a single exon (5). This has led to the suggestion that the whole Mage superfamily has evolved in mammals by retrotransposition followed by gene duplication from an ancestral gene (10). Accordingly, Necdin is not present in marsupials, and it was probably acquired by retrotransposition during the recent assembly of the Prader-Willi/Angelman syndrome region on the chromosome 15q in humans, an event that occurred 105–180 million yr ago (51). The ancestral *mage* gene is probably an ortholog of the *mage-D* genes, which are the only *mage* mammalian genes that contain introns (10).

Type II Mage proteins are widely expressed in many embryonic and adult tissues, particularly in the nervous system (2, 6, 29, 46, 53). These proteins also interact with the p75 neurotrophin receptor ($p75^{NTR}$) (5), which displays multiple functions in this tissue. To date these type II Mage proteins have been implicated in the regulation of cell cycle progression, cell differentiation, and apoptosis, acting as adaptors in multiple signal transduction pathways (54). The best characterized example of a mammalian type II Mage protein is Necdin, which was initially isolated from mouse embryonal carcinoma cells differentiated into neurons (42). The mouse *necdin* gene is predominantly expressed in postmitotic neurons (58), and when expressed ectopically, it suppresses proliferation (25) and triggers neuronal differentiation (31) in different cell lines. These latter effects seem to be mediated by the capacity of Necdin to interact with and block the transactiva-

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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tion domain of E2F-1 (56), a transcription factor necessary for G1/S phase progression that is capable of inducing apoptosis in postmitotic cells (22, 23). The absence of the *necdin* gene has been associated with the Prader-Willi syndrome, a neurogenetic disorder caused by the deletion of the 15q11-q13 segment of the paternal chromosome, which triggers mental retardation and other physiological alterations (45). Like Necdin, the type II Mage protein Mage-D1 is expressed in neurogenic areas of the developing rat and mouse nervous systems (29, 53), as well as in the mature rat brain (6). Mage-D1 can also suppress cell cycle progression, and it is able to promote $p75^{NTR}$-dependent apoptosis in sympathoadrenal cells (53). In addition, $p75^{NTR}$ has been shown to sequester Necdin or Mage-G1 through its intracellular domain ($p75^{ICD}$), thereby favoring the proapoptotic activity of E2F-1 in postmitotic neurons (33, 56).

While it is clear that type II Mage proteins are key elements in neurogenesis and proapoptotic signaling triggered by $p75^{NTR}$, our understanding of their function in the developing nervous system remains poor. The analysis of these proteins in mammals is complicated because of the large number of related genes with possible redundant functions. Indeed, only minor defects in the development of the nervous system have been observed in null-mutant mice for the *necdin* gene. Depending on the genetic background, these mice may die in the neonatal period due to apparent respiratory insufficiency that can be explained by abnormal neuronal activity within the putative respiratory rhythm-generating center. Alternatively, they may be viable and fertile simply displaying some functional alterations in the hypothalamus, and changes of behavior reminiscent of Prader-Willi syndrome (21, 45, 52). Therefore, a model system with fewer Mage proteins would facilitate the analysis of Mage function in development and adulthood.

In this study, we show that in the genome of nonmammalian species only one *mage* gene can be detected. Thus, we cloned the chicken Mage protein, CMage, whose pattern of expression is similar to that of $p75^{NTR}$ in the developing retina, ventral spinal cord, and dorsal root ganglia. Moreover, CMage shows functional similarities to the type II Mage protein Necdin, indicating that the chick may be a useful model system to further characterize the signal transduction pathways used by Mage proteins in the absence of functional redundancy.

MATERIALS AND METHODS

Chick embryos. Fertilized eggs from White Leghorn hens were obtained from a local supplier (Granja Santa Isabel, Cordoba, Spain), and they were incubated at 38.5°C in an atmosphere of 70% humidity. The embryos were staged according to Hamburger and Hamilton (24). Experimental procedures were approved by the CSIC animal ethics committee.

Primary antibodies. The rabbit anti-$p75^{NTR}$ polyclonal antiserum against the cytoplasmic domain of human $p75^{NTR}$ (Promega, Madison, WI) was used at a dilution of 1:500 for immunocytochemistry and 1:5,000 for Western blot analysis. The rabbit polyclonal antiserum (9992) against the intracellular domain of $p75^{NTR}$ was kindly provided by Moses Chao (New York University, New York, NY), and it was used at a dilution 1:1,000 for immunohistochemistry. The mouse polyclonal antibody obtained by immunizing mice with a $p75^{NTR}$ receptor-Fc chimeric protein (11), kindly provided by Alfredo Rodríguez-Tébar (CABIMER, Seville, Spain), was used at a dilution 1:500 for immunohistochemistry. The NC243 antiserum, raised against the 243 COOH-terminal amino acids of mouse Necdin (46), was used at a dilution of 1:2,000 for immunocytochemistry and immunohistochemistry, and a dilution of 1:20,000 in Western blots. The anti-FLAG monoclonal antibody (MAb; Sigma, St. Louis, MO) was used at 20 μg/ml for immunocytochemistry, at 30 μg/ml for immunoprecipitations and at 0.12 μg/ml in Western blots. The anti E2F-1 MAb KH95 (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:15,000 in Western blots. 5-Bromo-2′-deoxy-uridine (BrdU) was visualized with the G3G4 MAb (Developmental Studies Hybridoma Bank, Iowa City, IA) used at a dilution of 1:4,000. The mouse TuJ-1 MAb against neuron-specific βIII tubulin (Chemicon) was used at 1:2,000 dilution. The anti-Islet-1 MAb 40.2D6 (Developmental Studies Hybridoma Bank) was diluted 1:200 for immunohistochemistry.

cDNA probes for Southern blot. A 319-bp cDNA fragment (corresponding to bp 347–665 of clone ChEST965i23) was amplified by PCR from the pcDNA6-CMage-FLAG plasmid using specific oligonucleotides. This cDNA fragment, included in the region codifying for the MHD of CMage, was then labeled with digoxigenin-11-dUTP by random priming using DIG-High Prime (Roche, Basel, Switzerland) according to the manufacturer's instructions.

RNA probes for in situ hybridization. Complementary RNA probes for *cmage*, corresponding to bp 17–730 of clone ChEST965i23 [National Center for Biotechnology Information (NCBI) accession number BX934453] were generated by RT-PCR using a Pyrococcus furiosus (Pfu) DNA polymerase (Biotools, Madrid, Spain) and from a cDNA template derived from E4 eye/tectum. This PCR fragment was cloned into the pGEM-T Easy vector (Promega), and digoxigenin-labeled antisense riboprobes were obtained from linearized plasmid templates using Sp6 RNA polymerase (Roche).

Plasmids. The pRc/CMV-E2F-1 and the pRc/CMV-Necdin expression vector have been described previously (33, 56). The vector expressing $p75^{ICD}$ (pRc/CMV-$p75^{ICD}$-HA) was a generous gift of Yves-A. Barde (University of Basel, Basel, Switzerland). Green fluorescent protein (GFP) was expressed in the DF-1 cells by using the pEGFP-N1 plasmid (BD Biosciences, San Jose, CA). The coding sequence of CMage-FLAG corresponded to bp 17–754 of clone ChEST965i23 and was amplified with Pfu DNA polymerase (Biotools) from cDNAs derived from E4 eye/tectum using the following oligonucleotides: upstream primer (CACAAGCTTATGTCTCAGAGGAAGCGCAGC); downstream primer (CTCGAATTCCTACTTATCGTCGTCATCCTTGTAATCCGTGTGGCTTTGGCCTCG). The PCR amplification product of 786 bp contained a *Hin*dIII cleavage site at the 5′-end, and a FLAG tag sequence followed by a stop codon and an *Eco*RI restriction site at the 3′-end. This fragment was cut with *Hin*dIII and *Eco*RI, inserted in the *Hin*dIII/*Eco*RI site of pcDNA 6/V5-His-A vector (Invitrogen, Carlsbad, CA), and the integrity of the resulting expression vector (pcDNA6-CMage-FLAG) was confirmed by sequencing. The coding region of the *cmage* gene was cloned into the pGEM-T Easy vector (Promega) (see below) and referred to as pGEM-*cmage*. The pRFPRNAiC and pRFPRNAi Luciferase vectors (12) were provided by Stuart Wilson (University of Sheffield, Sheffield, UK). The pRFPRNAi CMage vector capable of suppressing *cmage* expression was constructed using the pRFPRNAiC plasmid following the procedures described previously (12). The target sequence used to interfere with the *cmage* mRNA corresponded to bp 168–189 of clone ChEST965i23. Similar results were obtained when the sequences corresponding to bp 174–195, 58–579, or 666–687 of clone ChEST965i23 were used (data not shown).

Southern blot analysis. Genomic DNA from E5 chick embryos (10 μg) was digested with *Cai*I (Fermentas) and separated on agarose gels. As positive controls, *Eco*RI/*Hin*dIII-digested pcDNA-CMage-FLAG plasmid (0.77 ng) and *Cai*I-digested pGEM-*cmage* (0.23 ng) were used, these digestions yielding 0.1 ng of DNA corresponding to the *cmage* cDNA or the *cmage* gene sequences, respectively. The DNA was transferred onto Hybond-N+ nylon filters (GE Healthcare) in 10× SSC (1× SSC contains 15 mM sodium citrate, 150 M NaCl; pH 7.0), and UV-cross linked (UV Stratalinker 2400, Stratagene). The

filters were hybridized overnight at 47°C with the digoxigenin-labeled probes (see above) using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) following the protocol recommended by the manufacturer. The filters were then washed at low stringency, twice in 2× SSC/0.1% SDS at room temperature for 5 min and once in 2× SSC/0.1% SDS at 57°C for 1 h. The probes were detected following the protocol described by the manufacturer and the filters were exposed to Hyperfilm ECL (GE Healthcare).

Cell culture. DF-1 chicken fibroblast cells and N1E-115 neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS) (Invitrogen) at 37°C in a water-saturated atmosphere containing 5% CO_2. N1E-115 neuroblastoma cells and DF-1 cells were both seeded at 30,000 cells/cm^2 and maintained for 24 h in DMEM/10% FCS. The cells were transfected with Lipofectamine 2000 (Invitrogen), and the transfected cells were induced to differentiate by adding 2% DMSO (Sigma) to the culture medium 24 h after transfection (30). The cells were then fixed or prepared for Western blot analysis 48 h after transfection. In experiments to quantify apoptosis, N1E-115 neuroblastoma cells were grown on 12-mm coverslips (Menzel-Gläser, Braunschweig, Germany) coated with 500 μg/ml poly(D-L)ornithine.

In vivo BrdU treatment. Eggs were opened at their blunt end, and 40 μl of a solution containing 10 mg/ml BrdU (Roche) prepared in phosphate-buffered saline (PBS) was applied to the chorioallantoic membrane. Subsequently, the eggs were sealed and returned to the incubator. Embryos were killed 1 h after BrdU treatment.

In situ hybridization. In situ hybridization was performed as described previously (44), performing all steps under RNase-free conditions. Chick embryos of the ages specified were fixed for 4–8h at 4°C in 4% paraformaldehyde (PFA), incubated overnight at 4°C in 100 mM sodium phosphate buffer containing 30% sucrose, and then embedded in the OCT compound Tissue-Tek (Sakura, Torrance, CA). Cryosections (12 μm) were collected on 3-aminopropyl-trimethoxysilane-coated slides (Sigma), postfixed for 15 min in 4% PFA, and then carbethoxylated for 30 min in PBS containing 0.1% active diethylpyrocarbonate (Sigma). Sections were equilibrated for 5 min in 5× SSC and then prehybridized for 2 h at 60°C in 5× SSC containing 50% formamide (Fluka, Seelze, Germany) and 50 μg/ml tRNA (Roche). Hybridization was performed at 60°C overnight in the same solution containing 400 ng/ml of digoxigenin-labeled probes. Prior to hybridization, the riboprobes were denatured for 5 min at 80°C and then cooled on ice. Following hybridization, the sections were washed three times for 1 h in 2× SSC at room temperature (RT), 2× SSC at 65°C, and 0.1× SSC at 65°C. Slides were then incubated for 1 h in 0.5% blocking reagent (Roche) prepared in 150 mM NaCl, 100 mM Tris·HCl pH 7.5 (NT), and the localization of the bound riboprobes was detected by incubating overnight at 4°C with an AP-coupled antidigoxigenin antibody (Roche) diluted 1:5,000 in 0.5% blocking reagent prepared in NT. The slides were then washed twice in NT and equilibrated in 50 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris·HCl pH 9.5 (MNT). The antibody was visualized using an alkaline phosphatase substrate (338 μg/ml nitro blue tetrazolium, 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate; Roche) in MNT buffer. Finally, the color reaction was stopped by washing the slides in a 10 mM Tris·HCl pH 8.0 solution containing 1 mM EDTA.

Immunohistochemistry. Immunohistochemistry was performed as described previously (38). In brief, embryos were fixed for 4 h at 4°C in 4% PFA incubated overnight at 4°C in 100 mM sodium phosphate buffer containing 30% sucrose and embedded in the OCT compound Tissue-Tek (Sakura). Cryosections 12 μm thick were permeabilized for 30 min at RT in the presence of 0.1% Triton X-100 (Sigma) in PBS (PBS-T). The sections were blocked for 1 h with 10% normal goat serum (NGS) in PBS-T and subsequently incubated overnight at 4°C with the primary antibody in 1% NGS/PBS-T. After five washes with PBS-T, the sections were incubated for 1 h at RT with a Cy2-conjugated goat anti-rabbit IgG (H+L) antibody (Jackson Immunoresearch, West Grove, PA) diluted 1,000-fold or an Alexa Fluor 594 goat anti-mouse IgG (H+L) antibody (Molecular Probes) diluted 1:1,000. Sections were finally washed five times in PBS-T and once in PBS alone, and the labeled sections were mounted in 50% glycerol in PBS. The sections that were immunolabeled for BrdU were first subjected to DNA denaturation by incubating for 30 min with 2 N HCl/0.33 × PBS at RT, which was neutralized by 3 × 15-min washes with 0.1 M Na borate (pH 8.9) and two 5-min washes with PBS-T. Images were acquired with a Leica (Nussloch, Germany) TCF-4D confocal microscope and used directly to create the figures.

Coimmunoprecipitation. To detect the E2F-1 complexes, combinations of pRc/CMV-Necdin, pcDNA6-CMage-FLAG, pRc/CMV-E2F-1, and pRc/CMV-p75^{ICD}-HA plasmids were transfected in N1E-115 neuroblastoma cells using Lipofectamine 2000 (Invitrogen). These cells were grown in P60 petri dishes at an initial density of 6.0×10^4 cells/cm^2, and they were induced to differentiate with 2% DMSO as described above. The cells were then lysed in a Potter microhomogenizer with 250 μl of lysis buffer containing: 20 mM Tris (Roche) pH 7.5, 100 mM NaCl (Merck, Darmstadt, Germany), 5 mM $MgCl_2$ (Merck), 0.5% Triton X-100 (Sigma), 0.5 mM EDTA (Merck), 0.5 μg/ml DNase I (Roche), and 1× protease inhibitor mix (Roche). After incubating the lysates for 10 min at 4°C, they were centrifuged at 13,000 *g* for 10 min at 4°C, and 50 μl of the supernatant from each extract was mixed with 50 μl of 2× Laemmli's buffer and boiled for 5 min (Input samples). The rest of the supernatant was incubated with 30 μg/ml anti-FLAG-specific MAb for 2h at 4°C followed by incubation with 20 μl (bed-volume) of protein A/G Sepharose (Santa Cruz Biotechnology) for 1 h at 4°C. Immunoprecipitates were washed three times with 500 μl of lysis buffer, mixed with 20 μl of 2× Laemmli's buffer lacking 2-mercaptoethanol, and boiled for 5 min.

Western blot. Total cell extracts from 1.5×10^5 cells, or 10-μl immunoprecipitates were separated by SDS PAGE on 11% acrylamide gels and transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were incubated for 1 h with 2% ECL *Advance* blocking agent (ECL Advanced Western Blotting Detection Kit) (GE Healthcare Europe, Munich, Germany) in PBS containing 0.1% Tween 20 (PBT) (Sigma), and incubated for 2 h at room temperature with the appropriate antisera in blocking buffer. After being washed the membranes five times in PBT, they were incubated for 1 h at room temperature with a peroxidase-conjugated Affinipure goat anti-rabbit IgG antibody diluted 1:1,660,000 (Jackson Immunoresearch) or a goat anti-mouse IgG horseradish peroxidase-conjugated antibody at 1:500,000 (Bio-Rad) in blocking buffer. Finally, they were washed again as above, and the protein bands were visualized using ECL Advanced Western Blotting Detection Kit (GE Healthcare Europe).

5′- and 3′-rapid amplification of cDNA ends. 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed with the BD SMART RACE cDNA Amplification Kit (BD Biosciences) using cDNA obtained from whole E3 chick embryos following the manufacturer's instructions. This cDNA was amplified with the Advantage 2 PCR Enzyme System (BD Biosciences) using *cmage*-specific oligos corresponding to bp 480–507 of clone ChEST965i23. The sequences of these oligos were TATGGGAGTTCCTGCGCCGGCTCCGGGT for 3′-RACE and ACCCGGAGCCGGCGCAGGAACTCCCATA for 5′-RACE.

Genomic DNA amplification. Amplification of the coding sequence of the *cmage* gene was performed with Pfu DNA polymerase (Biotools) ($n = 2$) or with CertAMP (Biotools) ($n = 2$) from E3 whole chick embryo genomic DNA using the oligonucleotides corresponding to the 5′- and 3′-ends of the coding region of *cmage* described above. The 5′-untranslated region (UTR) of *cmage* gene was amplified with Pfu DNA polymerase (Biotools) ($n = 2$) or with CertAMP (Biotools) ($n = 2$) from this same genomic DNA using the primers corresponding to bp 1–17 and bp 68–87 of the ChEST868j13 clone (NCBI accession number BU381672). The PCR amplified products were cloned into the pGEM-T Easy vector and sequenced.

Cell death analyses. N1E115 cells (6×10^4) were transfected with the pEGFP-N1 expression vector (BD Biosciences), together with different combinations of pRc/CMV-p75^{ICD}-HA, pRc/CMV-E2F-1, and pRc/CMV-Necdin, or pcDNA6-CMage-FLAG (1 μg each) using Lipofectamine 2000 (Invitrogen). The plasmid quantities were adjusted to 5 μg with pBlueScript (Stratagene, La Jolla, CA). Cells were induced to differentiate as described previously (33). To quantify apoptosis, the DNA of PFA-fixed N1E115 cells was labeled with 1 μg/ml bisbenzimide (Sigma), and the number of pyknotic nuclei was established. Cells were counted on a Nikon E80i microscope using an oil immersion ×60 objective with phase contrast and epifluorescence illumination, and an average of 500 cells were analyzed per coverslip. The means ± SE from at least three independent experiments are shown, and the statistical differences were analyzed by Student's *t*-test.

Database searches. Tblastn searches were performed in the nonredundant DNA database available at the NCBI database using the Mage homology domain of mouse Necdin (amino acids 116–280; NCBI accession number: BAA11183) or CMage (amino acids 57–221, Fig. 1*C*). We ran tblastn searches using the BLOSUM-62 substitution matrix and the default values for the gap costs (existence: 11; extension: 1). Blastn searches in the Biotechnology and Biological Sciences Research Council (BBSRC) ChickEST database (http://www.chick.umist.ac.uk) were performed using the ChEST965i23 se-

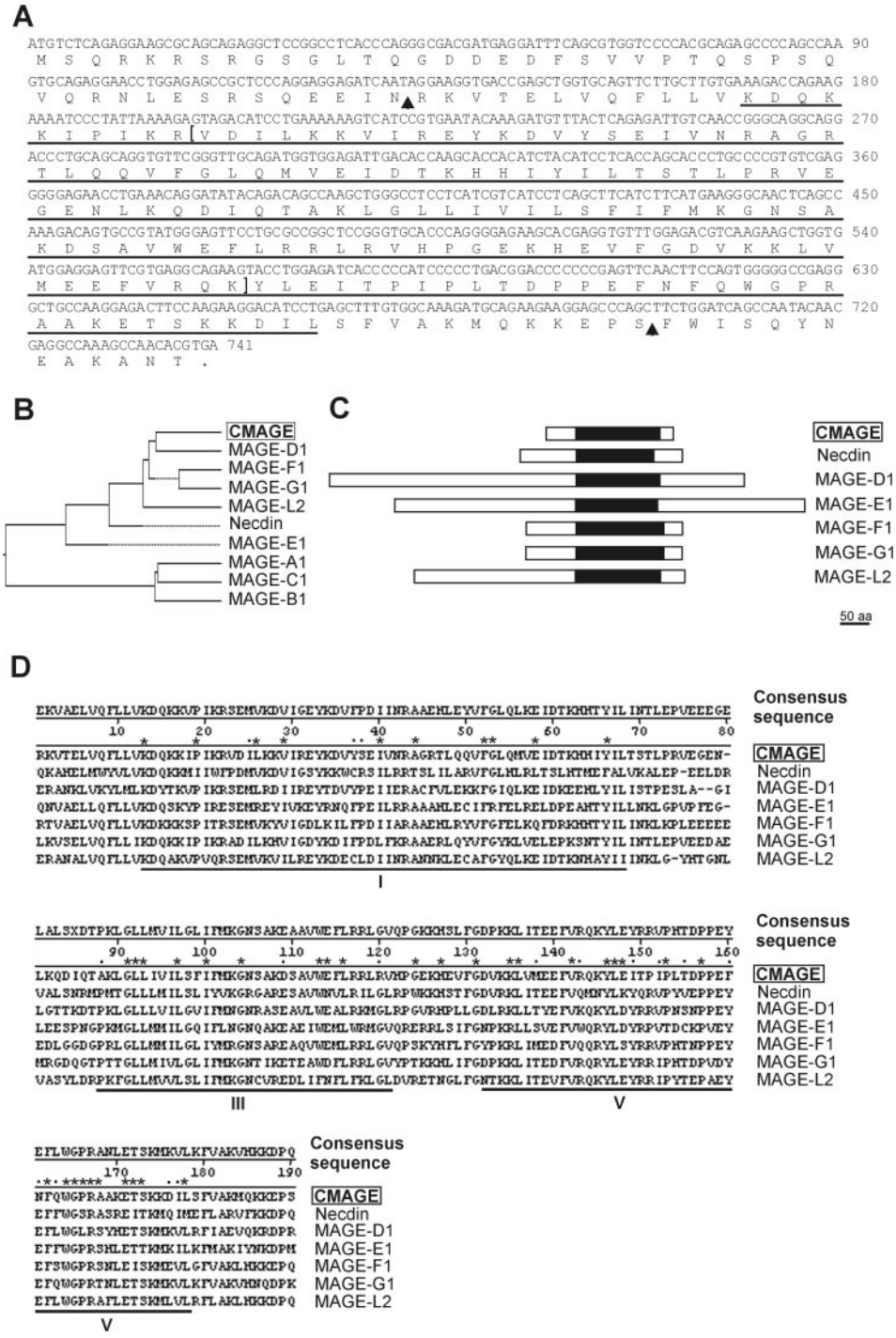

Fig. 1. Phylogenetic relationship between chicken melanoma antigen (CMage) and the type II mammalian Mage proteins. *A*: coding sequence of *cmage* and deduced amino acid sequence of CMage. The sequence corresponding to its Mage homolog domain (MHD), as proposed by Ref. 5, is underlined. Sequence between arrows correspond to that shown in *D* and Fig. 3*A*. Brackets indicate boundaries of homology between CMage and the hypothetical Mage protein (NCBI accession number XM_424083) predicted by automated computational analysis from a genomic region contained in the sequence with NCBI accession number NW_098113 (see text). *B*: phylogenetic tree obtained by sequence alignment of CMage with representative human Mage proteins. Note that CMage shows higher homology to the type II Mage proteins Mage-D1, Mage-E1, Mage-F1, Mage-G1, Mage-L2, or Necdin than to the type I Mage proteins Mage-A1, Mage-B1, or Mage-C1. *C*: scheme showing structural similarity of CMage to the human type II Mage proteins Necdin, Mage-D1, Mage-E1, Mage-F1, Mage-G1, and Mage-L2 (black boxes represent the MHD). *D*: sequence alignment performed by the CLUSTAL method to identify contigous regions of homology among the MHDs of CMage, and human Necdin, Mage-D1, Mage-E1, Mage-F1, Mage-G1, and Mage-L2. Regions I, III, and V are underlined, corresponding to the MHD previously described (5). Asterisks show Mage conserved residues, as proposed by Ref. 5, in CMage. The Mage conserved residues proposed by Ref. 5 that are not present in CMage are shown as dots.

quence as the query. Blastn searches were run using the BLOSUM-62 substitution matrix and the default values for gap and nucleotide mismatching. Blastn searches in the chicken genome database from NCBI were performed using the sequence between bp 185–679 of ChEST965i23, encoding the *cmage* MHD, following default settings.

Multiple sequence alignments. The Mage domains were aligned using the CLUSTAL method (MegaAlign package software). The high degree of homology between the proteins was revealed by the tblastn search ($E < 8e^{-45}$). Pair alignments were performed by the Lipman-Pearson method (MegaAlign package software).

RESULTS

Cloning of CMage and its gene. To identify chicken homologs of Mage proteins in mammals, we performed tblastn searches of *Gallus gallus* sequences in the nonredundant DNA database available at NCBI using the Mage homology domain of mouse Necdin (amino acids 116–280; NCBI accession number: BAA11183). This search identified a single expressed sequence tag (EST) cluster containing three sequences (ChEST965i23, NCBI accession number BX934453; ChEST970m21, NCBI accession number BX930943; and ChEST297b18, NCBI accession number CR354280) These sequences encode a putative Mage protein containing 246 amino acids (Fig. 1*A*) with an expected molecular mass of 28.5 kDa. We refer to this protein as CMage (for chicken Mage; NCBI accession numbers ABI98817 and ABI98818). Alignments between CMage and representative members of the different human Mage subfamilies indicated that CMage is highly related to the type II family (Fig. 1, *B–D*).

Part of the genomic sequence corresponding to the *cmage* gene, flanked by two gaps of uncertain length, is contained in a genomic contig with NCBI accession number NW_098113. From their sequence, five small exons can be deduced corresponding to bp 219–580 of the ChEST965i23 sequence (equivalent to bp 2738–2817, 2917–3011, 3127–3206, 3293–3335, and 3437–3500 from the genomic contig mentioned above). We have sequenced the whole region of the *cmage* gene encoding CMage, identifying four additional exons in the 5′-end and two in the 3′-end (NCBI accession number DQ983362). Therefore, from the abovementioned sequence 11 exons encompass the whole *cmage* gene, corresponding to bp 1–14, 167–210, 309–404, 597–660, 897–976, 1079–1173, 1278–1357, 1444–1486, 1588–1650, 1941–2055, and 2157–2219 (Fig. 2*A*), being the coding sequence of *cmage* comprising exons 2 through 11 (Fig. 2, *A* and *B*). Ten small introns (from 86 to 290 bp in size) distributed throughout the sequence were detected in the *cmage* gene, in accordance with the reduced size of the chicken genome known to be one-third that of a typical mammal (8). All the introns obey the GT-AG rule of splice junctions (Fig. 2*B*).

Blastn searches of the chicken genome using the region of *cmage* encoding the MHD of CMage did not yield any additional sequences, indicating that the chicken genome contains only a single *mage* gene. This conclusion was empirically confirmed by Southern blot analysis of chicken genomic DNA using a specific cDNA probe from the MHD region of *cmage*. Low stringency hybridization of the latter probe resulted in a unique band (Fig. 2*C*).

A hypothetical CMage protein (NCBI accession number XM_424083) was previously predicted by automated computational analysis of a genomic region contained in the genomic contig mentioned above. This hypothetical protein derives from part of intron 4 and exons 5 to 9 of the *cmage* gene sequence, followed by two exons included in the chicken ESTs ChEST561j8, ChEST911e13, and ChEST183i21. These latter EST sequences are almost identical to the chicken 6-phospho-fructo-2-kinase/fructose-2,6-biphosphatase 4 mRNA (NCBI accession number: NM_001030584). Moreover, the mRNA encoding this hypothetical CMage protein cannot be detected in the BBSRC ChickEST database. Indeed, we were unable to detect this mRNA by 3′- or 5′-RACE from E3 whole chick embryo or stage 36 heart cDNA (data not shown). Together, these data do not support the existence of this hypothetical protein.

mage gene family comprises single members in nonmammalian species. Although more than 10 different type II *mage* genes have been described in mammals (5), the genomes of *Drosophila*, teleost fish, and chicken (*G. gallus*) seem to contain only a single *mage* gene (7, 47, 50; this study). We therefore decided to verify whether this is also the case in other nonmammalian species. Using information available in the NCBI public database, we examined the existence of putative *mage* genes in the genome of birds (*Taeniopygia guttata*), amphibia (*Xenopus tropicalis*), fishes (*Danio rerio* and *Tetraodon nigroviridis*), echinoderma (*Strongylocentrotus purpuratus*), insects (*Drosophila melanogaster*, *Drosophila yakuba*, *Apis mellifera*, and *Anopheles gambiae*), plants (*Oryza sativa* and *Arabidopsis thaliana*), fungi (*Cryptococcus neoformans*), nematodes (*Caenorhabditis elegans*), and protists (*Entamoeba histolytica*: Table 1, Fig. 2*A*). These data indicate that the genomes from nonmammalian species contain only one *mage* gene (Fig. 2*A*), most probably the ortholog of the ancestral gene that gave rise to the Mage superfamily of proteins in mammals (Fig. 2*D*). Although it was proposed that the ancestral *mage* gene originally contained multiple exons (10), the coding sequences of the *Entamoeba histolityca* and *D. melanogaster mage* genes are contained in a single exon (Fig. 2*A*). Therefore, the ancestral *mage* gene was probably encoded by a single exon, and it has acquired introns during the course of evolution. Indeed, the number of introns in the *mage* genes in the different animal phyla seems to increase as they have evolved (Fig. 2*A*).

Alternative splicing of cmage transcripts. Unlike human *mage-D* genes (10), alternative splicing of exons in the *mage* genes from nonmammalian species seems to be a rare event (Fig. 2*A*). One exception is the *mage* gene from *D. rerio* (Fig. 2*A*) that encodes three different mRNA transcripts corresponding to full-length proteins (isoforms 1 and 3), as well as a hypothetical truncated Mage protein that lacks part of the MHD (isoform 2, NCBI accession number XP_708652) whose functional significance remains unclear (Fig. 2*A*). Therefore, we analyzed whether the *cmage* gene may also undergo alternative splicing. After performing a blastn search in the BBSRC ChickEST database with the ChEST965i23 sequence, we obtained 34 clones from different tissues of adult and embryonic chicken with a similar coding sequence, emphasizing that *cmage* is the only *mage* gene in the chicken genome. These EST sequences could be grouped into two major mRNA isoforms depending on the presence of intron 1 at their 5′-untranslated ends (Table 2). Thus, *cmage* isoform 1 retains this intron whereas in *cmage*

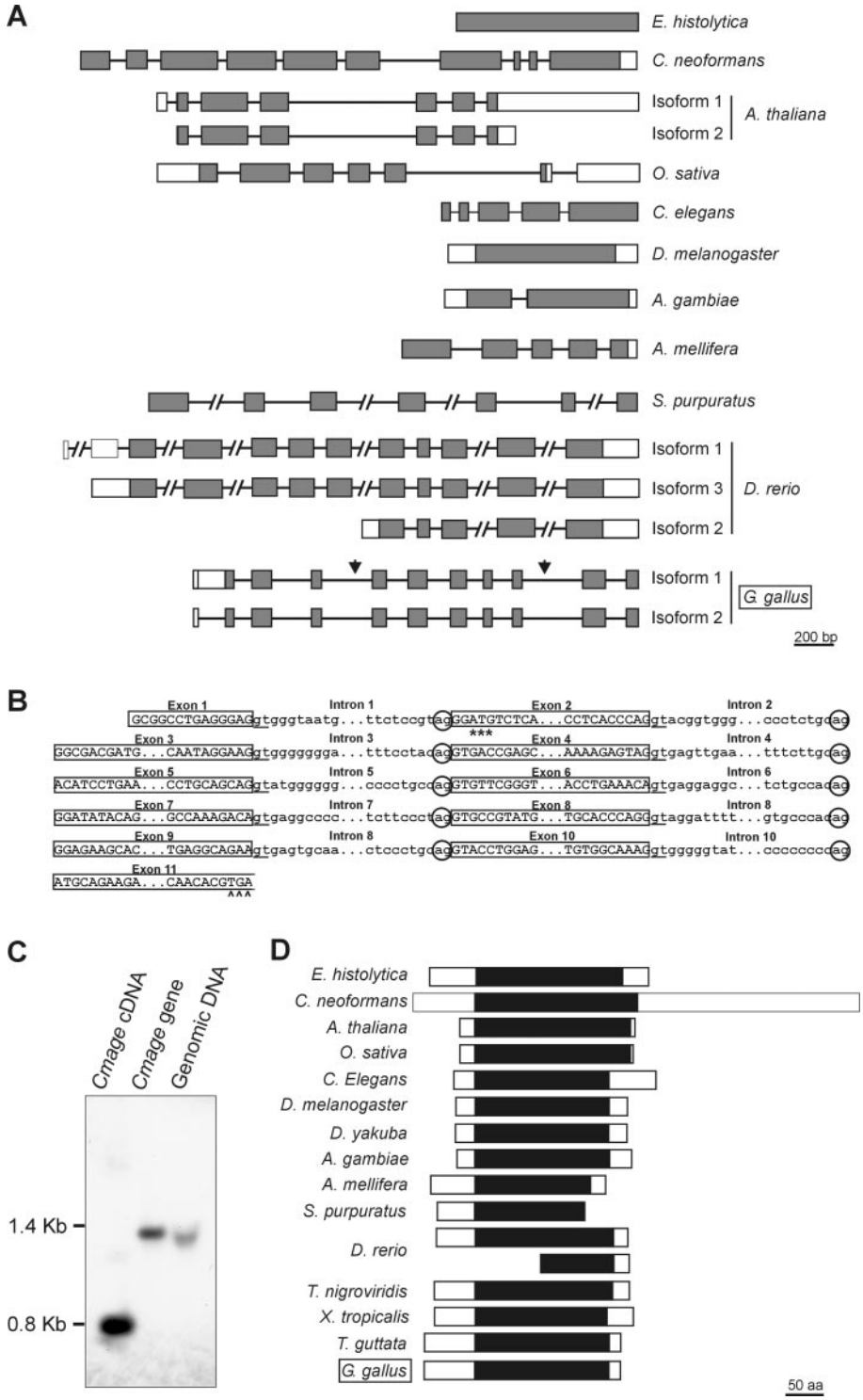

Fig. 2. Structure of the nonmammalian *mage* genes and proteins. *A*: genomic organization of the *mage* genes from the species indicated. Boxes represent sequences present in the mature mRNAs and lines introns. Coding regions are labeled in gray. Arrows indicate the partial sequence of *cmage* described previously (NCBI accession number NW_098113). Observe how the *cmage* isoform 1 is generated by retaining intron 1 in the 5′-untranslated region (UTR) of the mature mRNA. *B*: exon-intron boundaries corresponding to the coding sequence of the *cmage* gene. Circled nucleotides indicate the donor site junction, and the acceptor site is underlined. ***initial ATG; ^^^stop codon. *C*: Southern blot of genomic DNA obtained from embryonic day (E) 5 chick embryos (10 μg), digested with CaiI, and probed with a *cmage*-specific probe derived from part of the cDNA sequence encoding the MHD of CMage. A fragment of *cmage* cDNA (771 bp) and a fragment of *cmage* genomic DNA digested with *Cai*I (1,382 bp) were used as controls. The minor mobility shift with respect to the expected size observed in the *right* lane compared with the *middle* lane is probably due to distortions derived from the large amount of genomic DNA that was loaded into the gel. *D*: comparison of the Mage proteins from the species indicated with the MHD represented as black boxes.

isoform 2, this sequence is spliced out (Fig. 2*A*). Both these isoforms encode an identical protein, and they were readily detected by 5′-RACE from cDNA derived of E3 whole chick embryo (data not shown).

Conservation of the MHD during evolution. The MHD from nonmammalian proteins has been conserved during evolution, as seen by comparing the MHD sequences derived from the nonmammalian Mage proteins with the MHD of human Necdin, used in this study as a prototypical type II Mage protein (Fig. 3*A*). As described previously (5), five subdomains can be seen in these MHDs. The relationship between the aligned MHD sequences was represented by a phylogenetic tree diagram (Fig. 3*B*), revealing that the different MHD motifs largely recapitulate eukaryote evolution. This result is in agreement with previous studies showing phylogenetical conservation between *Drosophila* Mage and the mammalian Mage proteins (7, 50).

Table 1. *Mage proteins in nonmammalian species*

Species	Name	Protein	mRNA	Gene	Ref.
E. histolytica	hypothetical protein	XP_650381	XM_645289	NW_665401	(35)
C. neoformans	hypothetical protein	XP_571250	XM_571250	NC_006691[d]	(36)
A. thaliana	putative Mage protein	AAN86181	BT002348		DS
	unknown protein	NP_174733 (NP_849747)	NM_103197 (NM_179416)	NC_003070[d]	DS
	Mage	AAF40209[a,b]	AF234632		DS
O. sativa	putative Mage	BAC79510 (BAD31908)		AP003704 (AP006163)[d]	DS
	putative Mage	XP_476654	XM_476654		DS
C. elegans	hypothetical protein	AAC24293		U97406	(9)
D. melanogaster	RE25453p	AAL90350	AY089612		DS
	Mage CG10059-PA	NP_649702	NM_141445		(1)
	Mage	AAF89741[a]	AF170879		(50)
	CG10059-PA	AAG22204		AE003672	(1)
D. yakuba	Mage	AAR09713	AY231690		(16)
A. mellifera	Necdin-like 2	XP_623376	XM_623373	NW_625762[d]	DS
A. gambiae	ENSANGP00000026300	XP_559757	XM_559757	NT_O78268[d]	DS
S. purpuratus	similar to Necdin-like 2	XP_781230	XM_776137	NW_862332[d]	DS
D. rerio	Mage	AAO46100	AY228339		(7)
	Mage isoform 1	XP_687065[a]	XM_681973	NW_646916 (NC_007134)[d]	DS
	Mage isoform 2	XP_708652[a,c]	XM_703560	NW_646916 (NC_007134)[d]	DS
	Mage isoform 3	XP_708653[a]	XM_703561	NW_646916 (NC_007134)[d]	DS
	Ndnl2	AAH86747[a]	BC086747	NC_007134 (NW_646916)[d]	(55)
	Necdin-like 2	NP_942107[a,c]	NM_198812		(7)
T. nigroviridis	Full-length cDNA		CR690787		(27)
T. rubripes	Mage				(7)
X. tropicalis	Melanoma antigen family E, 1	NP_001016930	NM_001016930		DS
T. guttata	SB02047A2D05.f1		CK308710		DS
G. gallus	CMage	ABI98817/ABI98818	[e]	DQ983362	[f]

Protein, mRNA, and gene NCBI accession numbers are shown. [a]Minor discrepancies can be detected in the corresponding sequences, likely sequencing errors. [b]Truncated protein (due to truncated cDNA). [c]Truncated protein in the middle of the Mage domain (maybe as a consequence of alternative splicing). [d]Presence of introns. [e]See Table 2 [f]This study. Mage, melanoma antigen protein; CMage, chicken melanoma antigen protein; DS, direct submission.

Thus, Mage proteins seem to represent an ancient eukaryotic protein family.

Anti-Necdin antiserum NC243 specifically recognizes CMage. Since lower vertebrates appear to contain only a single *mage* gene in their genomes, the analysis of the signal transduction pathways mediated by type II Mage proteins might be simplified in these species. Birds shared a common ancestor with mammals ~310 million yr ago, at a phylogenetic distance not previously covered by other genome sequences (8). Therefore, these organisms are theoretically the best model system to study Mage protein function in higher vertebrates.

Table 2. *Chicken ESTs found in the BBSRC ChickEST database codifying for the isoforms of CMage described in Fig. 2A*

Isoform 1	Isoform 2	Partial cDNA
ChEST749f10	ChEST965i23	ChEST897h15
ChEST860c9	ChEST369g22	ChEST963113
ChEST356d4	ChEST398c2	ChEST706m9
ChEST383h18	ChEST582g19	ChEST463a6
ChEST91a4	ChEST868j13	ChEST891k23
ChEST410e8	ChEST860f7	ChEST366i8
ChEST407j20	ChEST486e21	
ChEST167a15	ChEST357i3	
ChEST278h3	ChEST583c3	
ChEST419i19	ChEST789113	
ChEST297b18	ChEST354f9	
ChEST173c23	ChEST976p20	
	ChEST970m21	
	ChEST968111	
	ChEST729a4	
	ChEST1005c5	

EST, expressed sequence tag; BBSRC, Biotechnology and Biological Sciences Research Council.

To further characterize CMage, we amplified its coding sequence from cDNAs obtained from E4 chick embryos using specific primers designed to add a FLAG tag in the COOH terminus of the molecule. This cDNA was subsequently cloned into a eukaryote expression vector. The flagged CMage protein was expressed in DF-1 cells, an immortalized chick fibroblast cell line, together with an RNA interference (RNAi) construct specific for either *cmage* or *luciferase*. Immunostaining performed with the NC243 anti-Necdin antiserum could be detected in most DF-1 cells cotransfected with *cmage* and *luciferase*-specific RNAi constructs, but not when *cmage* was coexpressed with the *cmage*-specific RNAi vector (Fig. 4*A*).

A 28.5-kDa band, corresponding to the expected molecular mass of CMage, was detected in total extracts from *cmage* transfected DF-1 cells or in DF-1 cells cotransfected with *cmage* and the *luciferase*-specific RNAi construct. In contrast, this band was not observed in the control untransfected DF-1 cells or in DF-1 cells cotransfected with *cmage* and the *cmage*-specific RNAi construct. This same band was also observed in extracts of E5 chick retinas (Fig. 4*B*). Together, these data indicate that the NC243 antiserum reliably recognizes chicken CMage.

High levels of CMage can be detected in vivo in regions that accumulate p75^{NTR} and are enriched in postmitotic neurons. The expression of *cmage* was analyzed in the chick embryo by in situ hybridization with a *cmage*-specific probe and by immunohistochemistry using the NC243 antiserum. At E4, the

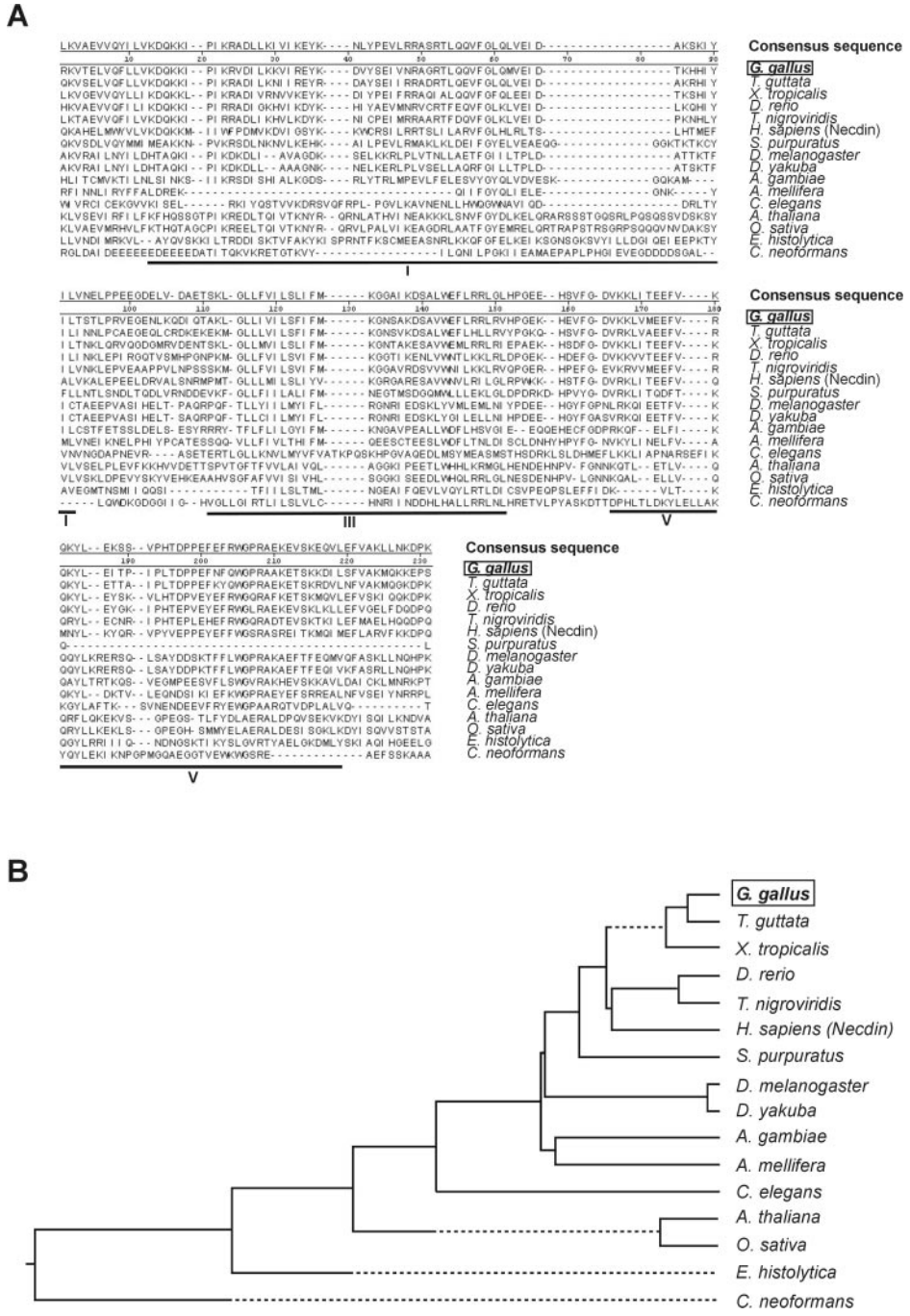

Fig. 3. Sequence comparison of the MHDs from known nonmammalian Mage proteins. *A*: an alignment of the MHDs from the nonmammalian Mage proteins together with human Necdin was performed by the CLUSTAL method to identify contigous regions of homology. The MHD subdomains I, III, and V described previously (5) are underlined. *B*: phylogenetic tree based on the homology between the Mage homology domains shown in *A*.

earliest stage analyzed, *cmage* mRNA was particularly enriched in the neural epithelium (Fig. 5*A*). Accordingly, strong levels of *cmage* mRNA were detected at different developmental stages in specific regions of the developing nervous system with the *cmage*-specific antisense probe (Fig. 5, *B, E, H,* and *K*), but not with a control probe containing the sense sequence of *cmage* (Fig. 5*B*). This analysis revealed that *cmage* was expressed strongly in the dorsal root ganglia at all developmental stages analyzed and in the ventral horn of the spinal cord between embryonic day (E) 4 and E6. The expression of *cmage* subsequently decreased in the ventral horn of the spinal cord as development proceeded and was virtually absent at E11. In addition, sympathetic ganglia also strongly expressed *cmage* mRNA at E6–E11. The expression of CMage in these neural structures was also analyzed by immunohistochemistry in adjacent brachial sections, and as expected, the distribution of CMage protein was highly coincident with that of its mRNA (Fig. 5, *C, F, I,* and *L*). Thus, CMage was enriched in cells located in the ventral horn of the spinal cord, the number of these cells diminishing as development proceeded, and it also accumulated in the dorsal root ganglia at all developmental stages studied. CMage was also detected in the sympathetic ganglia (data not shown).

The expression of *cmage* in the retina was also examined by in situ hybridization and immunohistochemistry. At E4, all retinal cells seem to express low levels of *cmage* mRNA and

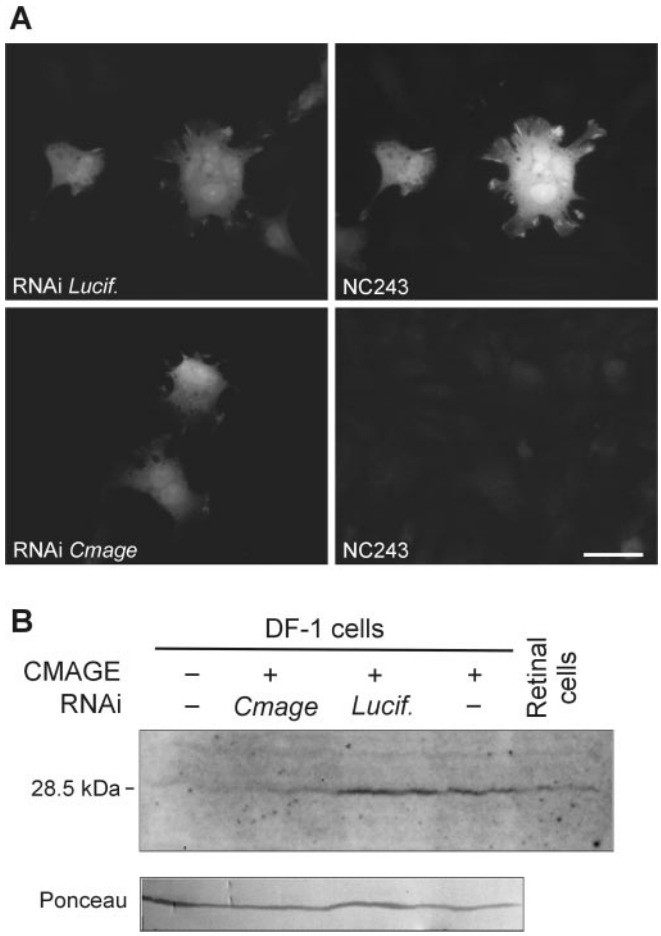

Fig. 4. Molecular characterization of CMage. *A*: CMage was expressed in DF-1 cells together with an RNA interference (RNAi) expression vector against Luciferase (RNAi *lucif.*) or an RNAi expression vector against *cmage* (RNAi *cmage*). After 24 h, the DF-1 cells were fixed and immunostained with the NC243 antibody raised against mouse Necdin (NC243), which was visualized with green fluorescence. RNAi vectors contained the coding sequence of red fluorescence protein and thus yielding red fluorescence in transfected DF-1 cells. Note how the NC243 antibody specifically recognized CMage in those cells transfected with the control RNAi expression vector. Bar: 15 μm. *B*: CMage was expressed in DF-1 cells together with an RNAi expression vector against *luciferase* (*lucif.*) or a RNAi expression vector against *cmage* (*cmage*). After 24 h, total extracts from DF-1 cells were obtained and subjected to Western blot analysis with the NC243 antibody. Note the 28.5-kDa band in extracts from DF-1 cells transfected with the RNAi expression vector against *luciferase* or with no RNAi expression vector. The CMage-specific 28.5-kDa band was highly reduced in the extracts derived from untransfected DF-1 cells or DF-1 cells transfected with the RNAi expression vector against *cmage*, demonstrating that the NC243 antibody specifically recognizes CMage. This same antibody recognized a specific 28.5-kDa band in total extracts from E5 chick retinal cells. The amount of protein extracted from DF-1 cells on the membranes was similar in all lanes as revealed by Ponceau S staining (Ponceau).

contain a little CMage protein, except the cells in the presumptive retinal ganglion cell layer (RGCL) that accumulated higher levels of CMage protein and its transcripts (Fig. 6, *A* and *B*). This expression pattern was maintained as development proceeded, and accordingly, most cells in the RGCL at E6–E8 strongly expressed *cmage* or contained high levels of CMage protein (Fig. 6, *D, E, G,* and *H*). At E11, cells containing high levels of *cmage* mRNA or the CMage protein could be observed in the RGCL, as well as in the most internal and external areas of the inner nuclear layer (Fig. 6, *J* and *K*).

To compare the expression pattern of CMage with that of $p75^{NTR}$, a protein known to interact with type II Mage proteins, we performed immunohistochemistry with a $p75^{ICD}$-specific antibody in adjacent sections. This analysis revealed that $p75^{ICD}$-specific immunostaining was present in the dorsal root ganglia and in cells located at the ventral horn of the spinal cord at all stages analyzed (Fig. 5, *D, G, J,* and *M*), coincident with the expression of CMage in these structures. A similar overlap in expression was also observed in the retina, where $p75^{NTR}$-specific labeling was observed in the RGCL at all stages analyzed, as well as in the most internal and external areas of the inner nuclear layer from E8 onward (Fig. 6, *C, F, I,* and *L*). Unlike CMage, at E11 $p75^{NTR}$ immunoreactivity was also observed in the inner plexiform layer. Finally, colocalization of CMage with $p75^{NTR}$ in the retina, the ventral horn of the spinal cord, and the dorsal root ganglia was confirmed by double immunostaining with a previously described mouse polyclonal anti-$p75^{NTR}$ antibody (11) together with the NC243 antiserum (Supplementary Fig. S1). (The online version of this article contains supplementary material.)

The areas where CMage were seen to be expressed are known to contain postmitotic neurons. To directly test whether CMage is mainly expressed by cells that cannot proliferate, chick embryos from different developmental stages were treated with BrdU for 1 h and then killed. Sections from these embryos were double immunostained with the NC243 antiserum and anti-BrdU. Most areas containing high levels of CMage immunolabeling excluded BrdU immunostaining (Fig. 7, *A* and *C*, and data not shown), indicating that the cells expressing CMage at high levels were postmitotic. Double labeling was also performed in these sections with the NC243 antiserum and the MAb TuJ-1, which specifically recognizes neurons (39). This analysis confirmed that the cells that express CMage at high levels in the dorsal root ganglia, the ventral horn of the spinal cord, and the retina are predominantly postmitotic neurons (Fig. 7, *B, D,* and *E*).

To define the identity of the CMage-positive neurons from the ventral spinal cord, double labeling with an anti-Islet-1 antibody was performed. Accordingly, many CMage-positive cells located in the ventral horn of the spinal cord coexpress Islet-1 (Fig. 7*F*), a spinal cord motoneuron-specific marker (17), demonstrating that motoneurons express CMage during the early stages of development. Interestingly, some CMage-positive neurons located in the ventral horn of the spinal cord do not show Islet-1 (Fig. 7*F*) nor $p75^{NTR}$ (Supplementary Fig. S1*C*) expression, indicating that CMage expression is not restricted to motoneurons.

CMage can interact with both E2F-1 and $p75^{ICD}$. To test whether CMage shows functional similarities to the mammalian type II Mage proteins, we compared it with Necdin, used in this study as a prototypical member of this protein family. Necdin has been shown to maintain neurons in a postmitotic state by binding to the transactivation domain of the E2F-1 transcription factor, thereby blocking its function and mimicking the function of Rb (56). Hence, we examined whether CMage also interacts with E2F-1 in differentiating N1E-115 neuroblastoma cells. Full-length $p75^{NTR}$ has been shown to interact with Necdin and Mage-G1 through its intracellular domain in differentiating N1E-115 neuroblastoma cells, dis-

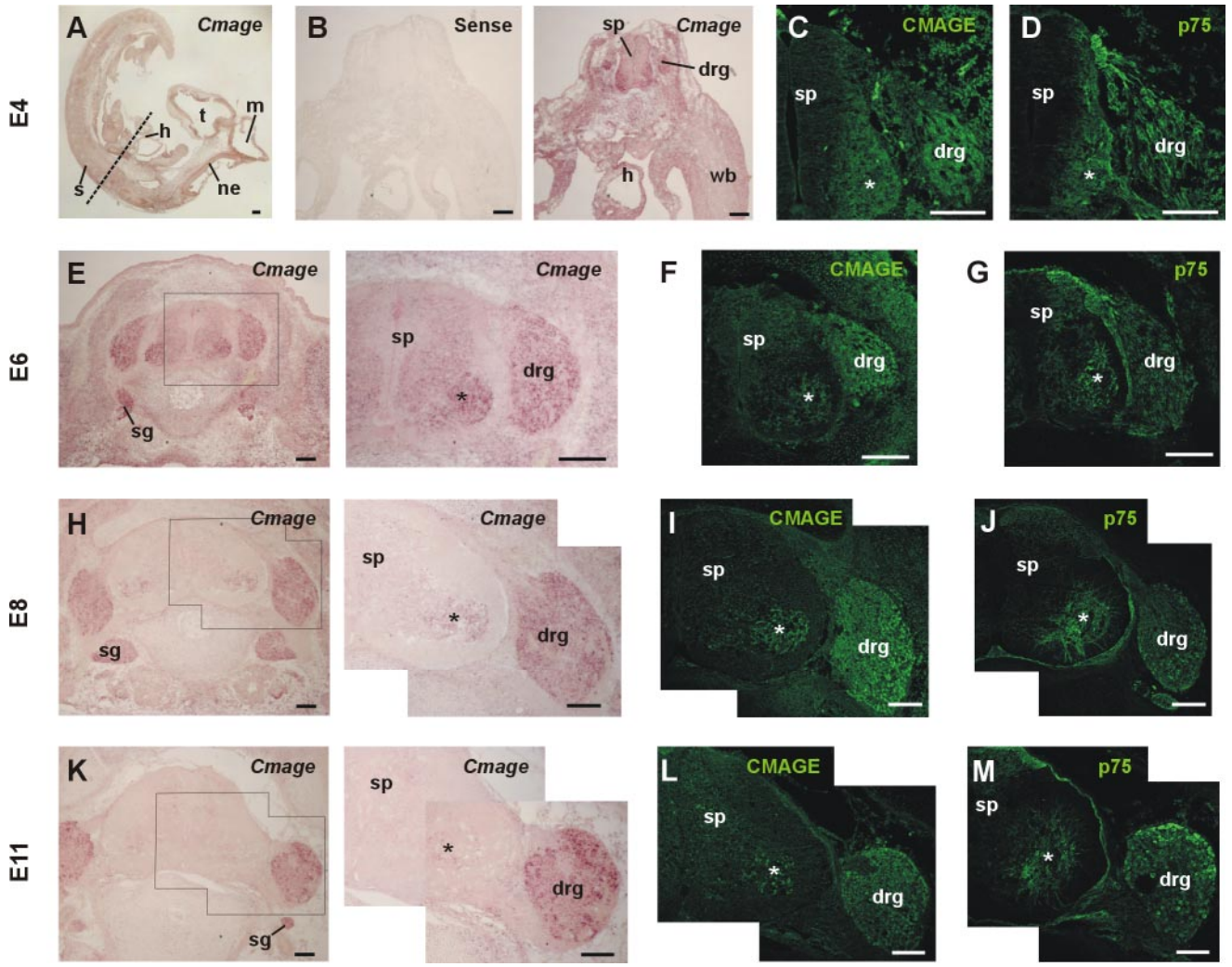

Fig. 5. Expression of *cmage* mRNA in the dorsal root ganglia and the ventral horn of the spinal cord visualized by in situ hybridization, and distribution of CMage and p75 neurotrophin receptor ($p75^{NTR}$) visualized by immunohistochemistry. A saggital section (12 μm, *A*) or transverse sections (*B–M*) from the brachial region (see dashed line in *A*) of chick embryos at the ages indicated were subjected to in situ hybridization using a *cmage*-specific probe (*cmage*) or a sense control probe (sense), or they were immunostained with the NC243 antiserum (CMage) or an antibody against the intracellular domain of $p75^{NTR}$ (p75). Observe the specific labeling for *cmage* or CMage of cells located in dorsal root ganglia (drg) cells at all developmental stages, coincident with $p75^{NTR}$ immunoreactivity. Cells in the ventral horn of the spinal cord (asterisk) were labeled for *cmage* or CMage. This labeling steadily decreased as development proceeds, whereas $p75^{NTR}$ immunolabeling was maintained through all the developmental stages analyzed. In *E, H, K*: panels at *right* represent higher magnification of the panels to the *left*. Sympathetic ganglia (sg) also expressed *cmage* at E6–E11. h, heart; m, mesencephalic vesicle; ne, neuroepithelium; s, somite; sp, spinal cord; t, telencephalic vesicle; wb, wing bud. Bar: 75 μm (*C, D*); 150 μm (*A, B, E–M*).

placing the interaction of these proteins with E2F-1 (33). Therefore, we also assessed whether the presence of $p75^{ICD}$ can prevent CMage from interacting with E2F-1.

To address these issues, we performed coimmunoprecipitation assays on cell extracts from N1E-115 neuroblastoma cells transfected with E2F-1 and CMage (or Necdin) alone or in the presence of $p75^{ICD}$ and induced to differentiate (30). An interaction between Necdin or CMage and E2F-1 was readily detected in the absence of $p75^{ICD}$ expression (Fig. 8, *A* and *B*), demonstrating that like Necdin, CMage can interact with E2F-1. The expression of $p75^{ICD}$ in differentiating N1E-115 neurons impaired the interaction between CMage and E2F-1 while a strong interaction of CMage with $p75^{ICD}$ was observed (Fig. 8*B*) similar to that observed for the interaction between Necdin and E2F-1 (Fig. 8*A*). Thus, we conclude that CMage can interact with $p75^{ICD}$ like other type II Mage proteins [including Mage-D1 (53), Necdin (33, 57), Mage-G1 (33), and Mage-H1 (57)]. Importantly, this association impaired the interaction of CMage with E2F-1, as previously shown for Necdin and Mage-G1 (33).

Interference of $p75^{ICD}$ with the E2F-1/CMage interaction facilitates apoptosis in differentiating N1E-115 neuroblastoma cells. The interaction of Necdin or Mage-G1 with E2F-1 abolishes the proapoptotic influence of the latter in differentiating N1E-115 neuroblastoma cells, whereas the expression of full-length $p75^{NTR}$ in these cells impedes these proteins from interacting with E2F-1, thereby favoring E2F-1-dependent neuronal death (33). We therefore studied whether CMage could mimic this effect, favoring E2F-1-dependent neuronal death in differentiating N1E-115 neuroblastoma cells. We quantified the percentage of condensed nuclei undergoing programmed cell death to assess the

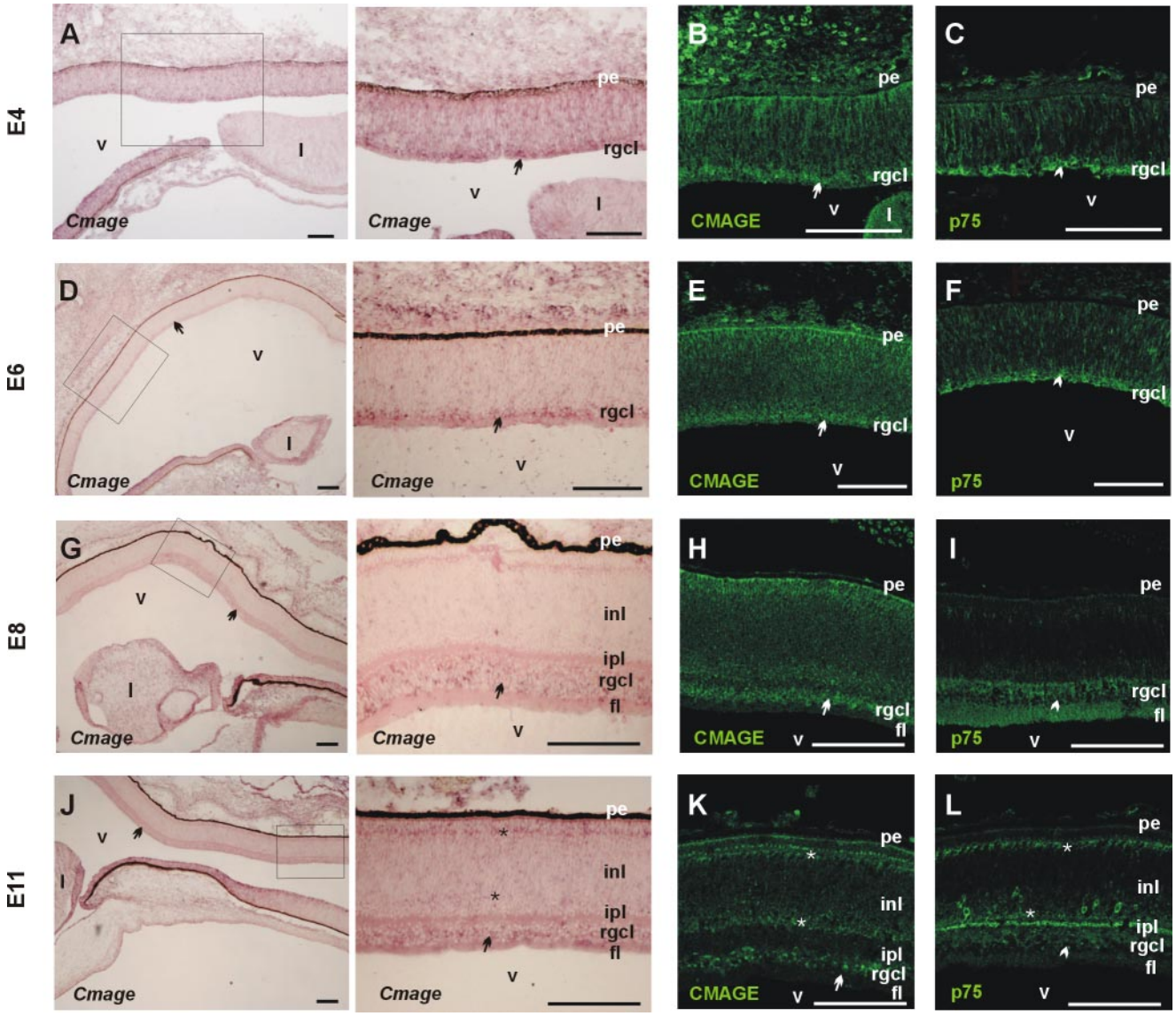

Fig. 6. Expression of *cmage* RNA in the retina visualized by in situ hybridization, and of CMage and $p75^{NTR}$ visualized by immunohistochemistry. Cryosections (12 μm) from chick embryos of the ages indicated were used for in situ hybridization using a *cmage*-specific probe (*cmage*) or immunostained with the NC243 antiserum (CMage) or an antibody against the intracellular domain of $p75^{NTR}$ (p75). Although at E4 most retinal cells seem to express *cmage* or contain the CMage product, its expression is higher in the presumptive retinal ganglion cell layer (rgcl, arrows in *A, B*). This pattern coincided with that of p75 intracellular domain ($p75^{ICD}$, arrowhead in *C*). At E6–E8, most cells expressing *cmage* or containing CMage protein were visible in the rgcl (arrows in *D, E, G, H*), coinciding with $p75^{ICD}$-positive cells in this region (arrowheads in *F, I*). At E11, most *cmage*- or CMage-expressing cells are confined to the rgcl (arrows in *J, K*) where $p75^{ICD}$-positive cells can also be observed (arrowhead in *L*). Cells positive for *cmage*, CMage, or $p75^{ICD}$ can also be observed in the most internal and external areas (asterisks in *K, L*) of the inner nuclear layer (inl). In *A, D, G, J*: panels at *right* represent higher magnification of the panels to the *left*. fl, Fiber layer; ipl, inner plexiform layer; l, lens; pe, pigmented epithelium; v, vitreous body. Bar: 75 μm (*A–C*); 150 μm (*D–L*).

extent of cell death observed in the differentiating N1E-115 neuroblastoma cells expressing CMage and E2F-1 alone or together with $p75^{ICD}$. As shown previously (33), the expression of E2F-1 in these cells dramatically increased the basal level of apoptosis (Fig. 9*B*). This cell death was partially prevented by the coexpression of CMage (Fig. 9*B*) in agreement with its capacity to bind E2F-1. The expression of $p75^{ICD}$ clearly neutralized the rescue from apoptosis by CMage (Fig. 9*B*), probably by impairing its interaction with E2F-1. A similar result was also obtained with Necdin instead of CMage (Fig. 9*A*). Therefore, we conclude that Necdin and CMage have the potential to inhibit E2F-1-dependent apoptosis, which can be prevented by their interaction with $p75^{ICD}$.

DISCUSSION

Mage proteins are expressed by single genes in nonmammalian species. In this study, we have detected the presence of single *mage* genes in all nonmammalian species for which the genomic sequences are available in the NCBI database, in accordance with earlier indications that only one *mage* gene can be detected in *Drosophila* and teleost fish (7, 47, 50). The presence of a single *mage* gene per genome in nonmammalian

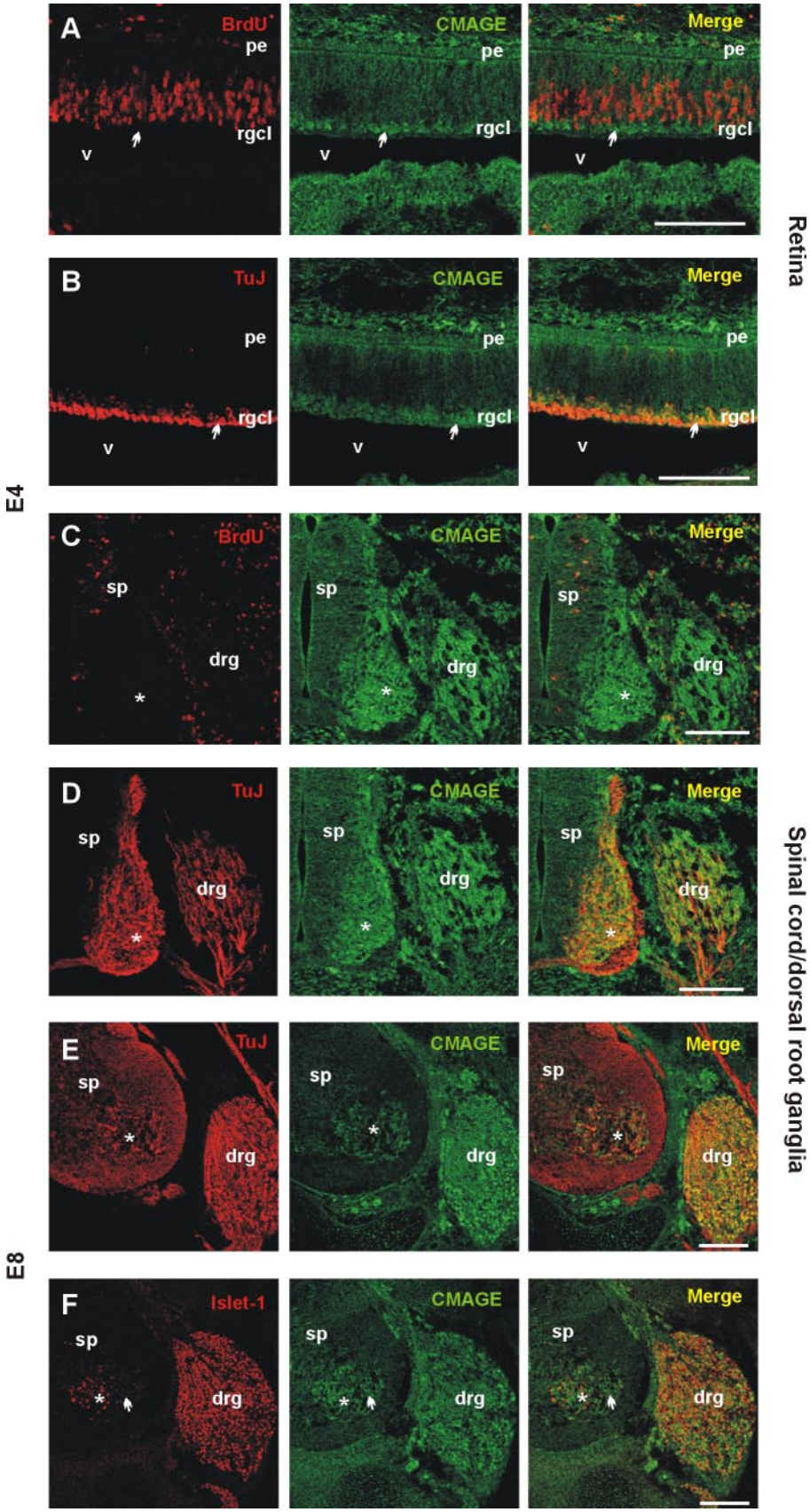

Fig. 7. CMage is expressed at high levels in postmitotic neurons. Cryosections (12 μm) from BrdU-treated chick embryos of the ages indicated were double immunostained with the NC243 antiserum (Mage, green) and anti-BrdU (BrdU), anti TuJ-1 (TuJ), or anti-Islet-1 (Islet-1) antibodies (red). Observe how most areas enriched in CMage labeling (arrows in *A, B*; asterisks and drg in *C–E*) were coincident with TuJ-1 immunoreactivity (*B, D, E*) but not with areas of BrdU incorporation (*A, C*). Although some CMage-positive cells located in the ventral horn of the spinal cord did not colocalized with Islet-1 immunoreactivity (arrows in *F*), many other cells coexpress Islet-1, a motoneuron-specific marker in the spinal cord, and CMage (asterisks in *F*). Drg cells also express Islet-1 (*F*). *Right* panels show merged images. Bar: 75 μm (*A–D*); 150 μm (*E, F*).

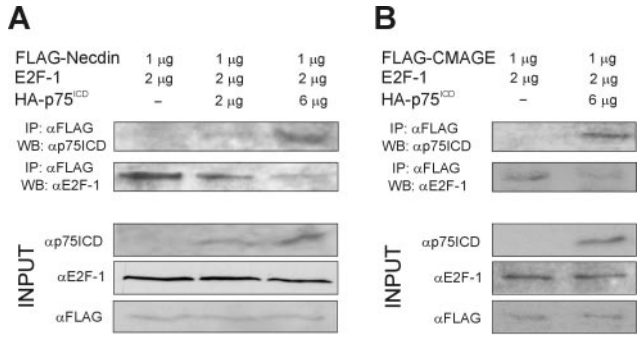

Fig. 8. Overexpression of $p75^{ICD}$ reduces the association of CMage with E2F-1 in differentiating N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells were transfected with different combinations of the expression vectors for FLAG-Necdin (*A*) or FLAG-CMage (*B*) and E2F-1, and with different amounts of HA-$p75^{ICD}$, before they were induced to differentiate with 2% DMSO. Cell lysates were immunoprecipitated (IP) with antibodies against the FLAG epitope (αFLAG), and immunoblotted (WB) with anti-$p75^{ICD}$ (αp75ICD) or anti-E2F-1 (αE2F-1) antibodies. Cell lysates were subjected to Western blotting for $p75^{ICD}$, E2F-1 and FLAG, (Input, *bottom*) to show the amount of these proteins in the lysates prior to immunoprecipitation.

species contrasts with the situation described in mammals, which contain dozens of different *mage* genes possibly generated by retrotransposition (10). Indeed, only genes belonging to the *mage-D* subfamily contain introns in their coding sequence, indicating that they are likely to be the orthologs of the common ancestor of all the *mage* genes. We believe that this ancestral *mage* gene may have lacked introns since the *mage* genes of *Entamoeba histolytica* and *D. melanogaster* do not contain introns in their sequences. The acquisition of introns seems to have played a role during the evolution of the *mage* genes, in accordance with the known importance of exon gain and loss during gene evolution (18).

Despite the presence of multiple exons in most nonmammalian *mage* genes, alternative splicing of exons is a rare event that usually takes place in noncoding regions. This indicates that only one Mage protein per genome is expressed in most nonmammalian species. Nevertheless, alternative forms of zebrafish *mage*-specific mRNA lacking the first exon can be found in the NCBI database. The analysis of the BBSRC ChickEST database indicated the existence of two main isoforms of *cmage* mRNA, similar to *mage* isoforms 1 and 3 from zebrafish, both encoding an identical form of CMage. *cmage* isoform 1 differs from isoform 2 in its 5′-UTR due to alternative splicing of intron 1 in the mature mRNA.

Structurally, most Mage proteins from nonmammalian species contain a conserved MHD in the center of the molecule. This domain has been conserved during evolution, and it is highly related to the MHD of the type II Mage proteins, in accordance with a previous study demonstrating that the Mage proteins from *Drosophila* and mammals are phylogenetically conserved (50). Most nonmammalian Mage proteins contain two small MHD flanking regions similar to human Necdin, Mage-G1, and Mage F1. Nevertheless, other mammalian Mage proteins have acquired additional domains in these flanking regions, as is the case of the Mage-D proteins that contain the so-called MHD2 and an "interspersed repeat domain" (5). Such motifs seem to have been acquired during the course of evolution of the mammalian genome as they are not present in nonmammalian Mage proteins.

CMage is a new member of the Mage family expressed in specific areas of the nervous system enriched in $p75^{NTR}$. In this study, we cloned the full-length coding sequence corresponding to the *cmage* gene, which contains eleven exons distributed over a 2.2-kb genomic fragment. In accordance with other nonmammalian species, *cmage* seems to be unique in the chicken genome since only a single band could be observed in low-stringency Southern blots of genomic DNA, and Blastn searches of the chicken genome did not yield any additional sequence to that of *cmage*. The cDNA cloned encodes CMage, which is structurally similar to the members of the type II Mage protein family.

CMage was specifically recognized by the NC243 antiserum directed against mouse Necdin, which detected a specific band of 28.5 kDa in extracts from DF-1 cells transiently transfected with CMage or from chick retinal cells and was able to immunostain CMage-expressing DF-1 cells but not control cells. Furthermore, immunostaining with NC243 in tissue sections yielded a similar pattern to that observed by in situ hybridization, again indicating that this antibody specifically recognizes CMage.

These experiments demonstrate that CMage was strongly expressed in developing areas enriched in projecting neurons such as the retinal ganglion cell layer, the ventral horn of the spinal cord, and dorsal root ganglia. Low levels of CMage expression were also detected in proliferating progenitor cells at the early stages of retinal development, in accordance with the finding that the *Drosophila* Mage protein may be expressed in mitotically active neural precursors such as neuroblasts and ganglion mother cells (47). Moreover, weak levels of this Mage protein were detected throughout the embryo, in accordance with the existence of ESTs from several specific tissues encoding CMage (Table 2). The areas where CMage was strongly expressed contain postmitotic neurons and express $p75^{NTR}$, known to interact with type II Mage proteins through its intracellular domain (5). Indeed, $p75^{NTR}$ is expressed by chicken retinal ganglion cells (60), dorsal root ganglia (26), and motoneurons (26, 43), and we confirmed that $p75^{NTR}$ is also expressed in these regions and colocalizes with CMage in most cells.

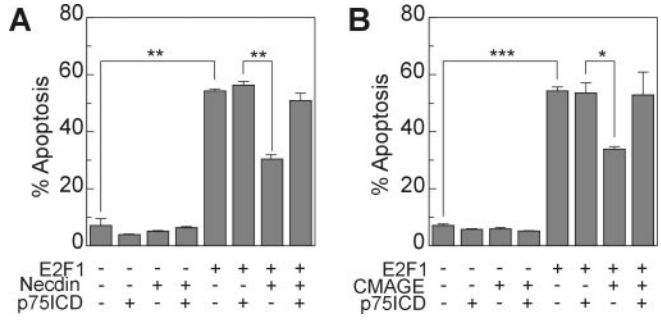

Fig. 9. Overexpression of $p75^{ICD}$ facilitates the proapoptotic activity of E2F-1 in differentiating N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells were transfected with combinations of expression vectors for E2F-1 (E2F1), HA-$p75^{ICD}$ (p75ICD), and FLAG-Necdin (Necdin) (*A*) or FLAG-CMage (CMage) (*B*), together with a green fluorescent protein (GFP)-expressing vector (1 μg each). Transfected cells were induced to differentiate with 2% DMSO for 24 h, fixed, and stained with bisbenzimide. The percentage of pyknotic nuclei as revealed by nuclear condensation after bisbenzimide staining was analyzed in GFP-positive cells and represented as percentages of apoptosis. $*P < 0.01$; $**P < 0.005$; $***P < 0.001$ ($n = 3$, Student's *t*-test).

CMage shows functional similarities with the type II Mage protein Necdin. In this study we present evidence of functional similarities between CMage and Necdin, based on the capacity of both proteins to interact with $p75^{NTR}$ and E2F-1.

$p75^{NTR}$ is able to transduce proapoptotic signals in response to ligand binding (20), which can be linked to alterations in cell cycle progression (37), and type II Mage proteins are known to interact with the intracellular domain of $p75^{NTR}$ (33, 53, 57), thus constituting a potential link between $p75^{NTR}$, cell cycle regulation, and apoptosis (37). To date, four different type II Mage proteins have been shown to interact with the intracellular domain of $p75^{NTR}$ in mammals: Necdin (33, 57), Mage-D1 (53), Mage-G1 (33), and Mage-H1 (57). Of these proteins, Necdin is predominantly expressed in postmitotic cells (2, 42, 58), and its pattern of expression in the developing mouse correlates with $p75^{NTR}$ expression (4), as occurs with CMage (this study). Through coimmunoprecipitation of total extracts from differentiating N1E-115 neuroblastoma cells cotransfected with $p75^{ICD}$ and CMage or Necdin, we demonstrated that the latter proteins are both able to interact with $p75^{ICD}$. These results stress the functional conservation between CMage and type II Mage proteins.

Besides its capacity to interact with $p75^{ICD}$, Necdin can suppress proliferation in several cell lines due to its capacity to repress the activity of the E2F-1 transcription factor (25, 56). Previous studies in differentiating N1E-115 neuroblastoma cells have demonstrated that full-length $p75^{NTR}$ can sequester Necdin and Mage-G1 at the cell membrane, preventing their interaction with E2F-1 (33). In this study, we have demonstrated that the binding of the $p75^{ICD}$ fragment to Necdin or CMage can prevent these latter proteins from establishing an inhibitory interaction with E2F-1.

The presence of functional E2F-1 in postmitotic neurons is known to trigger cell death through well-characterized mechanisms, including the stabilization of p53 levels or the induction of cdk1/cdc2 expression (22, 23), being the latter mechanism directly attenuated by Necdin (32). Furthermore, the activation of $p75^{NTR}$ by neurotrophins can provoke the release of $p75^{ICD}$, which is linked to the induction of apoptosis (19, 28, 49). We have demonstrated that releasing E2F-1 from its interaction with CMage or Necdin in the presence of $p75^{ICD}$ is capable of facilitating apoptosis of differentiating N1E-115 neuroblastoma cells, as occurs when Necdin is sequestered by full-length $p75^{NTR}$ in the cell membrane (33). In vivo, the lack of Necdin has been shown to trigger sensory defects derived from a loss of dorsal root ganglion neurons, probably due to apoptosis (3). Whether or not the mechanism used by Necdin to prevent this loss of sensory neurons is based on a mechanism dependent on $p75^{ICD}$/E2F-1 remains to be analyzed.

While Necdin and Mage-G1 have been reported to bind to $p75^{NTR}$ and E2F-1, other type II Mage proteins such as Mage-L2 do not bind to these proteins (33). This functional divergence raises the question as to whether the capacity of some Mage proteins to interact with $p75^{NTR}$ and E2F-1, preventing E2F-1 activity, was acquired during mammalian evolution. Conversely, this function may have existed in other phyla before the origin of mammals, and it was lost in some mammalian Mage members. In this study we demonstrate that this function seems to have arisen before mammals diverged from other phyla, as a nonmammalian Mage protein, CMage, is able to interact with $p75^{NTR}$ and E2F-1, and prevent the proapoptotic function of the latter.

In this study we have compared the function of CMage and Necdin. However, CMage appears to be more closely related to Mage-D1 in structural terms (Fig. 1*B*). Therefore, we cannot exclude that Mage-D1 may also be able to interact with E2F-1 and block its proapoptotic function, as previously shown for other type II Mage proteins (33). Mage-D1 has incorporated novel protein domains into its amino acid sequence during the course of mammalian evolution (5) and unlike Necdin and CMage, it is strongly expressed in proliferative neural subpopulations where $p75^{NTR}$ is absent (29, 53). These distinctive features of Mage-D1 indicate that this protein may have acquired additional functions to that of other type II Mage proteins during the course of mammalian evolution.

In conclusion, we believe that the functional similarity between CMage and Necdin coupled with the fact that only one *mage* gene is present in the chicken genome strengthens the idea that the chick represents a potentially useful model system to further analyze Mage protein function.

ACKNOWLEDGMENTS

We thank Ruth Diez del Corral, María-Jesús Latasa, and Mark Sefton for useful scientific comments and Yves-Alain Barde, Alfredo Rodríguez-Tébar, and Moses Chao for the gift of the $p75^{ICD}$ construct, the mouse anti-$p75^{NTR}$ polyclonal antibody, and the rabbit anti-$p75^{ICD}$ antiserum [9992], respectively. The anti-BrdU MAb G3G4, developed by Stephen J. Kaufman, and the anti-Islet1 MAb 40.2D6, developed by Thomas M. Jessell, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa.

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GRANTS

This work was supported by Ministerio de Educación y Ciencia Grants BMC2003-03441 and BFU2006-00805, by Fundación La Caixa Grant BM05-71-0, and by FUNDALUCE.

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