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

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**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/Magphin, 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<sup>NTR</sup>) (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 *necdn* 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-
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 p75NTR-dependent 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 p75NTR, 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 further characterization of 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-p75NTR polyclonal antisemur against the cytoplasmic domain of human p75NTR (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 p75NTR was kindly provided by Moses Chao (New York University, New York, NY), and it was used at a dilution of 1:1,000 for immunohistochemistry. The mouse polyclonal antibody obtained by immunizing mice with a p75NTR-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-Iset-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 pRcCMV-E2F-1 and the pRcCMV-Necdin expression vector have been described previously (33, 56). The vector expressing p75NTR (pRcCMV-p75, 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 (CACAGGTATGTCTCGAGGAAAGCGCAGC); downstream primer (CTCGAATTCCTAGCTTCCTATGCGTCTTTGCATAATCCGTGGCTTGGCTTGGCCTTCG). The PCR amplification product of 786 bp contained a HindIII cleavage site at the 5’-end, and a FLAG tag sequence followed by a stop codon and an EcoRI restriction site at the 3’-end. This fragment was cut with HindIII and EcoRI, inserted in the HindIII/EcoRI 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 Lu-cfase 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 pRFPRNAi 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 Call (Fermentas) and separated on agarose gels. As positive controls, EcoRI/HindIII-digested pcDNA-CMage-FLAG plasmid (0.77 ng) and Call-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 10x SSC (1 x SSC contains 15 mM sodium citrate, 150 M NaCl; pH 7.0), and UV-cross linked (UV Stratallinker 2400, Stratagene).
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).

**Culture cell.** DF-1 chicken fibroblast cells and NIE-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% CO2. NIE-115 neuroblastoma cells and DF-1 cells were both seeded at 30,000 cells/cm² 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, NIE-115 neuroblastoma cells were grown on 12-mm coverslips (Menzel-Gläser, Braunschweig, Germany) coated with 500 μg/mL poly(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 chorionicallantoic 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 4–8 h 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 carboxylated 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 65°C, and 0.1× SSC for 1 h. Following hybridization, the sections were washed two times for 15 min each. The antibody was visualized using an alkaline phosphatase/β-Naphtyl phosphate; Roche) in MNT buffer. Finally, the sections were 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 then washed two times with PBS-T. Images were acquired with a Leica (Nussloch, Germany) TCF-4D confocal microscope and used directly to create the figures.

**Immunoprecipitation.** To detect the E2F-1 complexes, combinations of pRc/CMV-Neodin, pcDNA6-CMAGE-FLAG, pRc/CMV-E2F-1, and pRc/CMV-p75ICD-HA plasmids were transfected in NIE-115 neuroblastoma cells using Lipofectamine 2000 (Invitrogen). These cells were grown in 500 petri dishes at an initial density of 6 × 104 cells/cm², 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 MgCl2 (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 2 h 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 × 105 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 1:1,600,000 (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 (RACE).** 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 cDNA-specific oligos corresponding to bp 480-677 of clone ChEST965123. The sequences of these oligos were TATGGGAGTCTTCTGGCCGCTCGGCTCGGT for 3′-RACE and ACCCCGAGCGCCGCGAGGACTCCCATA 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 1–7 and bp 68–87 of the ChEST686713 clone (NCBI accession number UC91672). The PCR amplified products were cloned into the pGEM-T Easy vector and sequenced.
Cell death analyses. N1E115 cells \((6 \times 10^4)\) were transfected with the pEGFP-N1 expression vector (BD Biosciences), together with different combinations of pRc/CMV-p75ICD-HA, pRc/CMV-E2F-1, and pRc/CMV-Necdin, or pcDNA6-CMage-FLAG (1 \(\mu\)g each) using Lipofectamine 2000 (Invitrogen). The plasmid quantities were adjusted to 5 \(\mu\)g 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 \(\mu\)g/ml bisbenzimide (Sigma), and the number of pyknotic nuclei was established. Cells were counted on a Nikon E80i microscope using an oil immersion \(\times 100\) 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 non-redundant 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. 1C). 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-
sequence 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. 1A) with an expected molecular mass of 28.5 kDa. We refer to this protein as CMage (for chicken Mage; NCBI accession numbers AB198817 and AB198818). 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′- 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. 2A), 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. 2B).

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. 2C).

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-phosphofructo-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), echinodera (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. 2A). These data indicate that the genomes from nonmammalian species contain only one mage gene (Fig. 2A), most probably the ortholog of the ancestral gene that gave rise to the Mage superfamily of proteins in mammals (Fig. 2D). Although it was proposed that the ancestral mage gene originally contained multiple exons (10), the coding sequences of the Entamoeba histolytica and D. melanogaster mage genes are contained in a single exon (Fig. 2A). 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. 2A).

**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. 2A). One exception is the mage gene from D. rerio (Fig. 2A) 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. 2A). 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
Isoform 2, this sequence is spliced out (Fig. 2A). 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. 3A). 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. 3B), 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).

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_008113). 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 Cai1, 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 Cai1 (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.
Table 1. Mage proteins in nonmammalian species

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<td>XM_559757</td>
<td>NT_O78268d</td>
<td>DS</td>
</tr>
<tr>
<td>T. guttata</td>
<td>CMage ABI98817/ABI98818</td>
<td>AB19817/AB19818</td>
<td>DQ983362</td>
<td>DQ983362</td>
<td>(8)</td>
</tr>
</tbody>
</table>

Protein, mRNA, and gene NCBI accession numbers are shown. *Minor discrepancies can be detected in the corresponding sequences, likely sequencing errors.  
+Truncated protein (due to truncated cDNA).  
*Truncated protein in the middle of the Mage domain (maybe as a consequence of alternative splicing).  
+Presence of introns.  
*See Table 2 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 approximately 310 million yr ago, at a phylogenetic distance not previously covered by other genome sequences. Mage proteins in nonmammalian species and in situ hybridization with a *cmage*-specific probe and by immunohistochemistry using the NC243 antiserum. At E4, the

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

<table>
<thead>
<tr>
<th>Isoform 1</th>
<th>Isoform 2</th>
<th>Partial cDNA</th>
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<tr>
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EST, expressed sequence tag; BBSRC, Biotechnology and Biological Sciences Research Council.
earliest stage analyzed, *cmage* mRNA was particularly enriched in the neural epithelium (Fig. 5A). 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. 5B). 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. 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 contiguous 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.
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 higher 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\textsuperscript{NTR}, a protein known to interact with type II Mage proteins, we performed immunohistochemistry with a p75\textsubscript{ICD}–specific antibody in adjacent sections. This analysis revealed that p75\textsubscript{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\textsuperscript{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\textsuperscript{NTR} immunoreactivity was also observed in the inner plexiform layer. Finally, colocalization of CMage with p75\textsuperscript{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\textsuperscript{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 postmitotic 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. 7F), 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. 7F) nor p75\textsuperscript{NTR} (Supplementary Fig. S1C) expression, indicating that CMage expression is not restricted to motoneurons.

CMage can interact with both E2F-1 and p75\textsubscript{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\textsuperscript{NTR} has been shown to interact with Necdin and Mage-G1 through its intracellular domain in differentiating N1E-115 neuroblastoma cells, dis-
placing the interaction of these proteins with E2F-1 (33). Therefore, we also assessed whether the presence of p75ICD 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 p75ICD and induced to differentiate (30). An interaction between Necdin or CMage and E2F-1 was readily detected in the absence of p75ICD expression (Fig. 8, A and B), demonstrating that like Necdin, CMage can interact with E2F-1. The expression of p75ICD in differentiating N1E-115 neurons impaired the interaction between CMage and E2F-1 while a strong interaction of CMage with p75ICD was observed (Fig. 8B) similar to that observed for the interaction between Necdin and E2F-1 (Fig. 8A). Thus, we conclude that CMage can interact with p75ICD 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 p75ICD 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 p75NTR 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
extent of cell death observed in the differentiating N1E-115 neuroblastoma cells expressing CMage and E2F-1 alone or together with p75ICD. As shown previously (33), the expression of E2F-1 in these cells dramatically increased the basal level of apoptosis (Fig. 9B). This cell death was partially prevented by the coexpression of CMage (Fig. 9B), probably by impairing its interaction with E2F-1. A similar result was also obtained with Necdin instead of CMage (Fig. 9A). Therefore, we conclude that Necdin and CMage have the potential to inhibit E2F-1-dependent apoptosis, which can be prevented by their interaction with p75ICD.

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).
species contrasts with the situation described in mammals, which contain dozens of different \textit{mage} genes possibly generated by retrotransposition (10). Indeed, only genes belonging to the \textit{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 \textit{mage} genes. We believe that this ancestral \textit{mage} gene may have lacked introns since the \textit{mage} genes of \textit{Entamoeba histolytica} and \textit{D. melanogaster} do not contain introns in their sequences. The acquisition of introns seems to have played a role during the evolution of the \textit{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 \textit{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 \textit{mage}-specific mRNA lacking the first exon can be found in the NCBI database. The analysis of the BBSC-NC ChE database indicated the existence of two main isoforms of \textit{cmage} mRNA, similar to \textit{mage} isoforms 1 and 3 from zebrafish, both encoding an identical form of CMage. \textit{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 protein of \textit{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\textsuperscript{NTR}.** In this study, we cloned the full-length coding sequence corresponding to the \textit{cmage} gene, which contains eleven exons distributed over a 2.2-kb genomic fragment. In accordance with other nonmammalian species, \textit{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 \textit{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 \textit{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\textsuperscript{NTR}, known to interact with type II Mage proteins through its intracellular domain (5). Indeed, p75\textsuperscript{NTR} is expressed by chicken retinal ganglion cells (60), dorsal root ganglia (26), and motoneurons (26, 43), and we confirmed that p75\textsuperscript{NTR} is also expressed in these regions and colocalizes with CMage in most cells.
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 p75NTR and E2F-1.

p75NTR 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 p75NTR (33, 53, 57), thus constituting a potential link between p75NTR, cell cycle regulation, and apoptosis (37). To date, four different type II Mage proteins have been shown to interact with the intracellular domain of p75NTR 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 p75NTR expression (4), as occurs with CMage (this study). Through coimmunoprecipitation of total extracts from differentiating N1E-115 neuroblastoma cells co-transfected with p75ICD and CMage or Necdin, we demonstrated that the latter proteins are both able to interact with p75ICD. These results stress the functional conservation between CMage and type II Mage proteins.

Besides its capacity to interact with p75ICD, 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 p75NTR 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 p75ICD 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 p75NTR by neurotrophins can provoke the release of Necdin. However, CMage appears to be more closely related to Mage-D1 in structural terms (Fig. 1B). 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 p75NTR 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.

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CHARACTERIZATION OF THE CHICKEN Mage PROTEIN


