p34cdc2 and mitotic cyclin expression in the developing quail neuroretina

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ABSTRACT After an initial proliferation phase, neurons of the central nervous system (CNS) of higher eukaryotes remain postmitotic during their entire lifespan. This requires that a very stringent control be exerted on the cell division apparatus, whose molecular mechanisms remain quite elusive. Here we have used quail neuroretina as a model to study the control of cell division in the developing CNS. In vertebrates, embryonic neuroretinal cells (NR cells) stop their proliferation at different times depending on the cell type. Most NR cells in the quail embryo become postmitotic between E7 and E8. To acquire a better understanding of the molecular events leading to quiescence in NR cells, we have analyzed the expression of cdc2 and of two activators of p34cdc2: cyclin A and cyclin B2 in the developing neuroretina. We report that these three proteins are downregulated between E7 and E9, suggesting that a common mechanism could block their transcription in differentiating neurons. We also report, using an immunohistochemical approach, that p34cdc2 downregulation is correlated with the appearance of the microtubule-associated protein tau. These results strongly suggest that inhibition of cdc2 gene expression is closely linked to the achievement of terminal differentiation in neurons. However, we also show that postmitotic ganglion cells precursors begin to synthesize the early neuronal differentiation marker B-tubulin while p34cdc2 is still detectable in these cells, suggesting that p34cdc2 or a closely related kinase could play a role in some "young" postmitotic neurons.

KEY WORDS: retina, cdc2, cell cycle, differentiation, cytoskeleton

Introduction

Embryonic development is characterized by an initial burst of cell proliferation, leading from a single cell to a mature multicellular organism. After this period, many cells stop dividing and enter terminal differentiation (Potten and Loeffler, 1980). This entry into a quiescent state needs to be tightly regulated in order to obtain a functional organism. In the nervous tissue of higher eukaryotes, almost all neuronal cells, after the initial phase of proliferation, become definitively quiescent (Oppenheim, 1991). The molecular mechanisms involved in this proliferation arrest are still poorly understood.

The Ser/Thr protein kinase p34cdc2, which plays a central role in the cell cycle, is considered as a major target of the molecular mechanisms involved in the establishment of quiescence in neuronal precursors. Indeed, deregulation of the mechanisms monitoring the activity of p34cdc2 can lead to apoptosis in higher eukaryotic cells (Fotedar et al., 1995; T. Shimizu et al., 1995). Several laboratories have shown that intracellular p34cdc2 concentration is downregulated during the development of the nervous system, suggesting that p34cdc2 is accumulated in proliferating neuroblasts and disappears in mature neurons (Krek and Nigg, 1989; Hayes et al., 1991; Okano et al., 1993). Rb knock-out mice exhibit profound alterations in the development of both the peripheral and central nervous systems, suggesting that this gene plays an important role in nerve cell differentiation (Lee et al., 1994). In addition, transient cotransfection experiments strongly suggest that the transcription factor E2F-1 regulates cdc2 expression in cycling cells through its binding to a high affinity response element, this transactivating effect being inhibited by p105RB (Dalton, 1992; North et al., 1996). Surprisingly, recent in vivo

Abbreviations used in this paper: BrdU, bromodeoxyuridine; CAIL, carbonic anhydrase II; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CNS, central nervous system; L, lens; INL, inner nuclear layer; NR, neuroretina; ONH, optic nerve head; PCNA, proliferation cell nuclear antigen; PE, pigmented epithelium; PMSE, phenylmethylsulfonyl fluoride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; VH, virucide humor.

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However, the precise molecular mechanisms underlying cdc2 downregulation during cell differentiation remain obscure.

Among the biological models available to study the control of cell proliferation in the CNS, the avian neuroretina is one of the best characterized systems (see e.g. Altschuler et al., 1991; Layer and Willbold, 1991; Lillien, 1994). Neurons of the avian retina are organized in concentric layers, each having its own differentiation time-course. The first cells to undergo terminal differentiation are the ganglion cells. They form an external layer of proliferating precursors, and as early as embryonic day E3, they become postmitotic and rapidly migrate to their definitive location close to the vitreous humor where they form the innermost cell layer of the retina (Fujita and Horii, 1963; Kahn, 1973; Spence and Robson, 1989; Harris, 1993) (see Fig. 1). Ganglion cells are also the first cells of the retina to express the early neuronal differentiation marker β₃-tubulin (Moody et al., 1989; Watanabe et al., 1991). Following ganglion cells, the other cell types differentiate with a precisely ordered time-table, bipolar cells differentiating last (Altschuler et al., 1991). In addition, central to peripheral as well as dorsal to ventral gradient of differentiation take place, the posterior pole undergoing differentiation first (Halfter et al., 1985; Prada et al., 1991).

We have recently shown that cdc2 transcription is downregulated around E7 in the quail embryo neuroretina, and that at least two transcription factors are involved in this down-regulation (North et al., 1996). However, since the various retinal cell types exhibit different kinetics of differentiation, it was important to study cdc2 expression in the developing retina at the cellular level with respect to the control of cell proliferation.

This paper describes the pattern of expression of p34cdc2 and associated cyclins A and B2 in the embryonic quail neuroretina, based on Western blotting and immunohistochemistry experiments. We report that p34cdc2, cyclin A and cyclin B2 gradually disappear between E7 and E9 with a similar time-course, suggesting that a common regulatory mechanism may control the amount of these proteins in retinal cells. Moreover, the detection of p34cdc2 by immunohistochemical methods, together with the identification of proliferating cells using bromodeoxyuridine (BrdU) incorporation, indicates that between E5 and E10, p34cdc2 accumulates in proliferating retinoblasts but not in postmitotic cells. In contrast, we have observed that, at E4, p34cdc2 is still present in early postmitotic ganglion cells, in regions which have already begun to differentiate as shown by the accumulation of the early differentiation marker β₃-tubulin. This result suggests that p34cdc2 could play a specific role shortly after the arrest of cell division in some postmitotic neurons during the early steps of differentiation.
Results

Expression of PCNA and differentiation markers in the developing retina

To evaluate the time-course of developmental events in the quail neuroretina, identical Western blots were probed with antibodies raised against the cell proliferation marker PCNA (see e.g. Sanders et al., 1993), and two differentiation markers: tau, a microtubule-associated protein, and β3-tubulin, a major component of microtubules in neurons. Tau is used as a marker of axonal elongation (Kosik, 1993; Pope et al., 1993; North et al., 1996); β3-tubulin, which is one of the earliest neuronal differentiation markers in the retina, is a neuron-specific form of β-tubulin which begins to accumulate during or shortly after terminal mitosis (Moody et al., 1989; Watanabe et al., 1991).

Results depicted in Figure 2 show that PCNA is abundant at E4-7, illustrating a high proliferation of retinal cells during this period, and gradually disappears the following days, being undetectable after E8. This indicates that the majority of retinal cells are postmitotic after E8. These observations are confirmed by the pattern of tau accumulation, which could be detected from E6-7, as differentiation progressively takes place. Multiple bands revealed with the anti-tau antibody, correspond to different embryonic tau isoforms with different phosphorylation patterns (Delacourte et al., 1990). Interestingly, β3-tubulin can be detected as early as E4 and is already very abundant at E5, indicating that a number of cells begin to differentiate early in the developing retina. Carbonic anhydrase II that remains at a constant level during the period studied (Linser and Moscona, 1984), was used as an internal standard for protein loading.

Synchronous downregulation of p34<sup>cdc2</sup> and cyclins

The behavior of the S phase marker PCNA during quail neuroretina development led us to examine whether these observations could be extended to cell division control genes, such as p34<sup>cdc2</sup> and mitotic cyclins A and B2, two regulators of p34<sup>cdc2</sup> kinase activity. To this end, a series of Western analyses were carried out using specific antibodies raised against these three proteins. Figure 3 shows that the amount of p34<sup>cdc2</sup> gradually decreases during the development of the retina, becoming almost undetectable after E8.

Interestingly, mitotic cyclins show a similar expression pattern as p34<sup>cdc2</sup>, disappearing around E7-9 (Fig. 3). This suggests that a co-ordinate regulation of the expression of p34<sup>cdc2</sup> and its partners takes place in the developing retina. The apparently more rapid decrease of cyclin B2 may be due to a shorter half-life in comparison with p34<sup>cdc2</sup> and cyclin A (Fig. 3). technical reasons such as film exposure length or small calibration errors could also explain this result.

Activity of p34<sup>cdc2</sup> kinase has been evaluated using histone H1 as a substrate in vitro. Partial purification of p34<sup>cdc2</sup> has been carried out using p13<sup>sek1</sup>-protein coupled to sepharose beads. This ligand binds to p34<sup>cdc2</sup> and other kinases, in particular p33<sup>cdk2</sup> (Gabrielli et al., 1992). As shown in Figure 4, the downregulation of p34<sup>cdc2</sup> and mitotic cyclins is paralleled by a drop of histone H1 kinase activity. Interestingly, this drop of kinase activity seems to occur slightly before the disappearance of p34<sup>cdc2</sup> and cyclins (compare Figs. 3 and 4).

Immunohistochemical analysis of cdc2 expression in the developing retina

The results presented so far suggest that arrest of cell division in the developing retina is correlated with the onset of terminal differentiation. In addition, the expression pattern of β3-tubulin is in agreement with previous observations showing that a number of ganglion cells have already undertaken differentiation while other cell types are still actively proliferating (Spence and Robson, 1989; Watanabe et al., 1991). To specify these points at the cellular level, we have performed an immunohistochemical analysis of PCNA, p34<sup>cdc2</sup>, β3-tubulin and tau expression between E4 and E10 (Fig. 5). S phase events have been detected using BrdU incorporation. All tissue sections analyzed in Figure 5 correspond to the central area of the retina located in the vicinity of the optic nerve head (Fig. 1).

At E4, BrdU incorporation (Fig. 5, upper panels) shows that in quail neuroretina a number of cells have already ceased to proliferate in the ventricular half of the retina. Most labeled cells are located in the vitreal half of the retina, however some isolated cells located close to the vitreous humor appear unlabeled, suggesting that these cells are also postmitotic. PCNA labeling confirms the results obtained with BrdU, showing that the most highly labeled cells are localized in the vitreal half of the retina at E4. A diffuse and
less intense PCNA labeling can be detected in non-proliferating areas (see e.g. Fig. 5, stage E4). This probably corresponds to quiescent cells where PCNA is located in the cytoplasm, as already described (Vriz et al., 1992). Interestingly, β3-tubulin (Fig. 5, lower panels) is already present near the vitreal side of the retina at E4. This cytoskeletal protein is most likely synthesized by the postmitotic cells that are already present in this area, as shown by BrdU incorporation. A faint β3-tubulin signal could also be observed near the ventricular side of the retina where most postmitotic cells are found (see arrow, Fig. 5). These observations are in good agreement with previous data obtained in chicken embryos, showing that the precursors of ganglion cells are located close to the ventricular side of the retina and cease to proliferate early in development (Spence and Robson, 1989; Layer and Willbold, 1991; Prada et al., 1991; Watanabe et al., 1991). These cells then begin to rapidly differentiate, as shown by the detection of β3-tubulin in the vicinity of the pigmented epithelium. They subsequently migrate to their definitive location close to the vitreous humor where they continue to differentiate, accumulating high amounts of β3-tubulin (Watanabe et al., 1991; see Fig. 1). Surprisingly, p34cdc2 labeling was found to be homogeneous in the whole retina, the protein being detected in proliferating area, as expected, but also in ventricular postmitotic cells.

At E6, BrdU incorporation and PCNA labeling in adjacent cell layers allow the identification of the postmitotic ganglion cell layer which remain unlabeled. Indeed at this stage all ganglion cell precursors have completed their migration from the ventricular side of the retina (Watanabe et al., 1991). BrdU is fading, while tau and β3-tubulin labeling is increasing near the vitreal side, reflecting continuation of neuronal differentiation. The result obtained with p34cdc2 at E6 indicates that, on average, it is less abundant as compared to E4, which is consistent with an increasing proportion of postmitotic cells in the developing retina. Almost no p34cdc2 labeling could be observed in the ganglion cell layer, which is also in agreement with the postmitotic state of these cells.

At E8, at which the stratification process of the retina has begun, BrdU incorporation, as well as PCNA and p34cdc2 labeling, are mainly localized in the inner nuclear layer (INL), corresponding to bipolar cells. The photoreceptor layer (outer nuclear layer) is unlabeled (Figs. 1C and 5), which indicates that these cells have completed their proliferation program.

At E10, very few cells incorporated BrdU, while no p34cdc2 labeling was detected, except in a few nuclei of the INL, illustrating that the vast majority of retinal cells are postmitotic at this stage. This observation was corroborated by the intense signal obtained with the antibodies specific for the differentiation markers tau and β3-tubulin.

Discussion

As an integral part of developmental processes, cell cycle regulation is expected to be tightly coupled to the induction of the

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Fig. 3. Western blot analysis of p34cdc2, cyclin A and cyclin B2 in the developing quail neuroretina. Three blots identical to those presented in Figure 2 were probed with anti-p34cdc2 (A), anti-cyclin A (B) and anti-cyclin B (C) antibodies. The detection system is the same as in Figure 2. Positions of molecular weight markers are indicated on the right.

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Fig. 4. Analysis of histone H1 kinase activity. (A) Left panel: protein complexes containing p34cdc2 were purified on p13ctc1 affinity beads from quail neuroretina protein extracts prepared at the indicated embryonic ages. p34cdc2 kinase activity towards histone H1 was measured as described in Materials and Methods. Position of histone H1 is indicated (arrow). Right panel: same experiment performed without histone H1 (negative control). (B) Coomassie blue staining showing position of histone H1 (arrow).
Fig. 5. Measurement of BrdU incorporation and analysis of the expression of PCNA, p34\textsuperscript{cdk2}, tau and \(\beta\)-tubulin by immunohistochemistry in the embryonic retina (E4-10). See the Materials and Methods for experimental details. Tissue sections correspond to the posterior pole of the retina. PE, pigmented epithelium, top; VH, vitreous humor, bottom. In some cases the pigmented epithelium has been removed (e.g. TUB/E6). A tubulin-positive cell located in the ventricular side of the retina is shown at E4 (arrow).
genetic programs which are expressed in differentiated cells. The precise molecular mechanisms which regulate the switch from a proliferative to a postmitotic differentiated state remain quite elusive in the nervous system. In this study, we have used the embryonic quail neuroretina as a model of neural development to examine the correlation between the arrest of cell proliferation and the onset of differentiation. To this end, we have analyzed in the developing quail neuroretina, the expression pattern of proteins which are known to be directly involved in either cell division control or neuronal differentiation. Recent observations suggest that the downregulation of p34\(^{cdc2}\) kinase activity, a major enzyme involved in the control of mitosis, is an important step which permits commitment to cell differentiation (Gaeta et al., 1991; Okano et al., 1993; Foteder et al., 1995; T. Shimizu et al., 1995). Certain aspects of the molecular mechanisms underlying this downregulation remain unknown, in part because they may vary depending on the cell type (Krek and Nigg, 1989; Welch and Wang, 1992). We have reported that cdc2 gene expression is downregulated during neuroretinal development (North et al., 1996). We show here that the protein p34\(^{cdc2}\) gradually disappears between E7 and E9 with the same timetable as cdc2 transcript (see North et al., 1996). This suggests that the amount of p34\(^{cdc2}\) is mainly controlled at the transcriptional level in the developing retina. p34\(^{cdc2}\) belongs to a family of related kinases, in particular cdk2 and cdk3, the two other CDK harboring the same PSTAIRE epitope as p34\(^{cdc2}\) (Meyerson et al., 1992), and the neuron-specific kinase cdk5, involved in neurite outgrowth (Meyerson et al., 1992; Nikolic et al., 1996). We have therefore used antibodies specifically directed against p34\(^{cdc2}\) to make sure that no other member of the Cdk family is revealed in Western blotting experiments. However, results obtained with anti-PSTAIRE and anti-cdk2 antibodies have shown that p33\(^{cdk2}\) has the same behavior as p34\(^{cdc2}\) in the developing retina (results not shown), which suggests that regulation of the expression of these two kinases is co-ordinated. Two other proteins, cyclins\(A\) and \(B\), which are activators of p34\(^{cdc2}\) kinase activity, disappear with the same kinetics as p34\(^{cdc2}\) (Fig. 3). This suggests that a common mechanism could monitor the intracellular concentration of these three proteins. Indeed, it has been recently reported that common response elements to transcription factors are found in the promoters of these genes (Zwicker et al., 1995; Brandeis and Hunt, 1996; North et al., 1996). Although the kinase activity of p34\(^{cdc2}\) has been measured on partially purified extracts probably containing other kinases like p33\(^{cdk2}\) (Gabrielli et al., 1992), the drop of histone H1 kinase activity observed between E6 and E7 (Fig. 4) may be at least in part contributed by the disappearance of p34\(^{cdc2}\) and associated cyclins. Inhibition of p34\(^{cdc2}\) and related kinases may also be due to specific inhibitors acting at the posttranslational level, like the p34\(^{cdc2}\) inhibitor p21. Indeed, p21 activity is induced by NGF in PC12 cells and in other cellular models of differentiation (Halevy et al., 1995; Skapek et al., 1995; Yan and Ziff, 1995; Bilion et al., 1996). Moreover, p105\(^{RB}\) is rapidly dephosphorylated between E6 and E7 in the developing quail neural retina (North et al., 1996), suggesting that cyclin-dependent kinases inhibitors (CDKI) are upregulated at this particular stage. It would therefore be of interest to study p21 expression in the developing retina, a speculative hypothesis being that p21, or a related CDKI, is involved in short term inhibition of p34\(^{cdc2}\) activity in the early stages of retinoblast differentiation, while repression of cdc2 gene expression by pocket proteins of the Rb family participates in the maintenance of long term quiescence in mature neurons (Okano et al., 1993; Lee et al., 1994; North et al., 1996).

In the embryonic retina, Western analysis shows that the level of the cell proliferation marker PCNA decreases dramatically after E8 (Fig. 2), indicating that most retinal cells are postmitotic at this stage. This result is in good agreement with previous \(^{(3}H\)thymidine incorporation studies (Spence and Robson, 1989; Prada et al., 1991). The levels of p34\(^{cdc2}\), cyclin A and cyclin B\(_2\) decrease gradually, paralleled by the accumulation of the microtubule-associated protein tau (Figs. 2 and 3). These observations suggest that the downregulation of cdc2 expression is correlated with neuronal differentiation. Immunochemical analyses were carried out to evaluate variations in the expression of selected proteins in single cells during neuroretinal differentiation. Kinetics of differentiation vary among the different cell types in the neural retina, with ganglion cells differentiating first and bipolar cells last (Altschuler et al., 1991; Layer and Willbold, 1991; Lillien, 1994). One could thus expect that retinoblasts will cease to express cell cycle genes at different embryonic stages, depending on the beginning of their own differentiation program. These studies confirm that ganglion cells differentiate early in development, expressing b3-tubulin as early as E4. Taken together, our observations indicate that cdc2 is expressed in proliferating cells and is downregulated in postmitotic cells between E6 and E10. Indeed, cdc2 downregulation is probably necessary to allow commitment to terminal differentiation (Kranenburg et al., 1995), whereas deregulation of cdc2 expression may lead to apoptosis (Foteder et al., 1995; T. Shimizu et al., 1995).

A different conclusion has however to be drawn from the results obtained at E4. Interestingly, at this stage, p34\(^{cdc2}\) is homogeneously distributed throughout the whole retina, including postmitotic ganglion cell precursors, the majority of which are still located in the vicinity of the ventricular side of the retina as shown by BrdU incorporation. This strongly suggests that at least in ganglion cell precursors, p34\(^{cdc2}\) is still accumulated in the early steps following cell division arrest. Numerous reports have shown that cdc2 gene expression is downregulated upon cell differentiation (Krek and Nigg, 1989; Hayes et al., 1991; Martinez et al., 1992; Okano et al., 1993; North et al., 1996). In differentiating neurons, a p34\(^{cdc2}\) related protein kinase is accumulated (Tsai et al., 1993); this kinase may play a role in neuronal differentiation by phosphorylating neurofilaments and the microtubule-associated protein tau (Lee and Cleveland, 1994); recent reports suggest that this activity corresponds, at least in part, to the cdk5 kinase (Nikolic et al., 1996). Activity of p34\(^{cdc2}\) itself in postmitotic cells has been reported in differentiating lens fiber cells (Gao et al., 1995), where it may participate to denucleation events and in early differentiating granule neurons of rat cerebellum (Hayes et al., 1991). The role of p34\(^{cdc2}\) in postmitotic retinal cells is unknown. This Ser/Thr kinase could possibly control microtubule dynamics in growing axons (Kranenburg et al., 1995); alternatively, p34\(^{cdc2}\) may participate in the induction of apoptosis in ganglion cell precursors that occurs during the development of the retina (Hugues and McLoon, 1979; Straznicky and Chehade, 1987). Identification of specific substrates of p34\(^{cdc2}\) in ganglion cells may help to understand the early events of neuronal differentiation in the retina and the central nervous system.
Materials and Methods

Animal strain
Quail (Coturnix coturnix japonica) fertile eggs were incubated at 37°C in a humidified atmosphere and submitted to periodic rocking motion.

BrDU incorporation
50 µl of BrDu solution (Sigma; 90 µg·ml⁻¹) was injected into a choanal lobe vein through a small hole made in the shell, which was then covered with an adhesive plastic tape to prevent dehydration. Eggs were further incubated for 2 h before dissection of the eyes for immunohistochemical detection.

Antibodies
- Anti-PCNA: mouse monoclonal (19F4, Boehringer); anti-p34Cdc2, mouse monoclonal (17, Santa Cruz), for Western blots, and rabbit polyclonal (Krek and Nigg, 1991), for immunohistochemistry; anti-CALI: rabbit polyclonal (Woods, et al., 1986); anti-tau: mouse monoclonal was a gift from Dr. A. Delacourte, see (Delacourte et al., 1990). Anti-ßIII-tubulin (TuJ1) was a gift from Dr. A. Frankfurter, see Moody et al. (1989); anti-BrDU: mouse monoclonal (Amerham); anti-cyclin A and anti-cyclin B2: rabbit polyclonal. A gift from Dr. E. Nigg.

Western blots
SDS-PAGE was performed following the standard technique of Laemmli (1970) with 5-20% polyacrylamide linear gradient gels. After transfer onto nitrocellulose (Schleicher and Schuell) and blocking with bovine serum albumin, the blots were incubated overnight at 4°C with the first antibody. The dilutions were as follows: anti-PCNA: 1:200; anti-p34Cdc2: 1:500; anti-CALI: 1:250; anti-tau: 1:500; anti-ßIII-tubulin: 1:500; anti-cyclin: 1:500. After incubation for 1 h in the appropriate peroxidase-linked secondary antibody, detection of chemiluminescence was made following the standard protocol described in the ECL user's guide (Amersham). Histone H1 kinase assay
100 µg of retinal protein extracts (S100 supernatants) were mixed with 20 µl of yeast p105-Cdc2 protein coupled to agarose beads (Brizuela et al., 1987) and 180 µl of beads buffer in eppendorf tubes. The beads were kept at 4°C for 30 min with continuous rocking. The beads were then washed three times with bead buffer and once with histone H1 buffer. The pellet was then resuspended in 65 µl of reaction buffer and incubated for 10 min at 30°C. The reaction products were then separated by SDS-PAGE (Laemmli, 1970) on a 15% acrylamide gel. The gel was then dried and radioactivity revealed by autoradiography.

Lysis buffer: 50 mM Tris (pH 7.4), 250 mM NaCl, 0.1% NP40, 1 mM DTT, 5 mM EDTA, 50 mM NaF, 50 mg·ml⁻¹ PMSF, 10 mg·ml⁻¹ TPCK, 1 mg·ml⁻¹ leupeptin, 1 mg·ml⁻¹ aprotonin, 10 mg·ml⁻¹ soybean trypsin inhibitor. Bead buffer: 50 mM Tris (pH 7.4), 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 10 mg·ml⁻¹ leupeptin, 10 mg·ml⁻¹ aprotonin, 10 mg·ml⁻¹ soybean trypsin inhibitor, 100 mM benzamidine. Histone H1 buffer: 50 mM Tris (pH 7.4), 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT, 60 mM β-glycerophosphate, 15 μM ATP, 0.5 μM protein kinase inhibitor (Sigma).

Reaction buffer: 5 µl γ[32P]ATP, 450 µl histone H1 buffer, 50 µl histone H1 (10 mg·ml⁻¹, Boehringer).

Immunohistochemistry
Eyes were removed from 4 to 10 day-old quail embryos (3 embryos for each day) and fixed in PBS, 4% paraformaldehyde for 2 h. The fixed tissues were dehydrated in ascending series of alcohol and butanol baths and then embedded in paraffin. 3 to 5 µm thick sections were mounted onto gelatin-treated glass slides and dried at 38°C for 16 h. After paraffin removal, sections were first incubated for 30 min in phosphate buffer saline (PBS) containing 1% normal goat serum and 1% bovine serum albumin; then the sections were incubated with the primary antibodies diluted in PBS containing 1% normal goat serum and 1% normal swain serum at 4°C for 16 h. The dilutions were as follows: anti-P53: 1/40; anti-p34Cdc2: 1:500; anti-tau: 1/250; anti-BrDU: 1/1. After washing, the sections were incubated for 3 h at room temperature in 1/20 diluted solutions of either sheep anti-mouse or donkey anti-rabbit immunoglobulins linked to horseradish peroxidase (Amersham) in PBS 1% normal goat serum containing 1% normal swain serum. Slides were rinsed twice in PBS (1 h) after each incubation step. The peroxidase activity was revealed by immersing the sections in PBS containing 0.25 M diaminobenzidine tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide (5 to 10 min).

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