

Newt Myotubes Reenter the Cell Cycle by Phosphorylation of the Retinoblastoma Protein

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Abstract. Withdrawal from the cell cycle is an essential aspect of vertebrate muscle differentiation and requires the retinoblastoma (Rb) protein that inhibits expression of genes needed for cell cycle entry. It was shown recently that cultured myotubes derived from the Rb^{-/-} mouse reenter the cell cycle after serum stimulation (Schneider, J.W., W. Gu, L. Zhu, V. Mahdavi, and B. Nadal-Ginard. 1994. *Science (Wash. DC)*. 264:1467–1471). In contrast with other vertebrates, adult urodele amphibians such as the newt can regenerate their limbs, a process involving cell cycle reentry and local reversal of differentiation. Here we show that myotubes formed

in culture from newt limb cells are refractory to several growth factors, but they undergo S phase after serum stimulation and accumulate 4N nuclei. This response to serum is inhibited by contact with mononucleate cells. Despite the phenotypic parallel with Rb^{-/-} mouse myotubes, Rb is expressed in the newt myotubes, and its phosphorylation via cyclin-dependent kinase 4/6 is required for cell cycle reentry. Thus, the postmitotic arrest of urodele myotubes, although intact in certain respects, can be undermined by a pathway that is inactive in other vertebrates. This may be important for the regenerative ability of these animals.

TERMINAL differentiation is associated with stable withdrawal from the cell cycle. In mammalian systems, the return of mature, differentiated tissue to an undifferentiated proliferating state is blocked since, in many tissues, a return to the cell cycle could in principle result in tumor formation or cell death. In contrast, in urodele amphibians such as the adult newt, reversal of differentiation is an integral part of their ability to regenerate limbs and other structures (Brookes, 1994; Okada, 1991). After amputation, epidermal cells from around the wound surface migrate across it to form the wound epidermis. The mesenchymal tissues beneath the wound epidermis dedifferentiate to produce blastemal cells, the proliferating and undifferentiated cells that are the progenitors of the new limb (Steen, 1968; Hay, 1959; Kintner and Brookes, 1984; Casimir et al., 1988). The capacity of newt myotubes to dedifferentiate was demonstrated directly by purifying myotubes formed from cultured newt limb cells, labeling them by injection of a lineage tracer, and implanting them beneath the wound epidermis of an early blastema (Lo et al., 1993). 1–3 wk after implantation, labeled mononucleate cells were found in the blastema, and their number in-

creased with time, indicating that the cells were proliferating. This experiment suggests that the local environment of the blastema stimulates newt myotubes to reenter the cell cycle and to reverse their differentiated state, thus raising a number of issues concerning the identity of the signals that stimulate dedifferentiation, as well as the underlying mechanisms that allow newt cells but not their mammalian counterparts to undergo this process.

Muscle has been a particularly informative system for studying how cells maintain the nondividing, differentiated state (Lassar et al., 1994; Olson, 1992). During differentiation, myoblasts exit from the cell cycle in the G₁ phase and fuse to form a multinucleate syncytium that expresses muscle-specific proteins and no longer responds to mitogens. It has been shown that this insensitivity is not caused solely by the down-regulation of cell surface receptors, nor by an irreversible alteration in the capacity of the nucleus to undergo DNA synthesis. The addition of mitogens such as EGF after cell cycle arrest but before receptor down-regulation provokes various intracellular responses, but it does not stimulate cell division (Endo and Nadal-Ginard, 1986; Olwin and Hauschka, 1988; Hu and Olsen, 1990). On the other hand, if myotubes are transfected with transforming viral proteins such as SV-40 large T antigen or adenovirus E1A protein, the myotube nuclei are induced to enter S phase (Endo and Nadal-Ginard, 1989; Iujvidin et al., 1990; Crescenzi et al., 1995).

These experiments with viral oncogenes suggest that the

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retinoblastoma (Rb)¹ protein might have a critical role in maintaining the postmitotic state because mutants of T antigen that are unable to bind Rb do not promote cell cycle reentry (Gu et al., 1993). This role of Rb has recently been demonstrated directly: myoblast cells derived from the Rb homozygous null (Rb^{-/-}) mouse form myotubes that express muscle-specific proteins, but they reenter S phase in response to serum (Schneider et al., 1994). The Rb protein is a regulator of the G₁-S restriction point of the cell cycle and acts through the E2F family of transcription factors that control the expression of several genes whose products are required for entry into S phase (Nevins, 1992; LaThangue, 1994; Riley et al., 1995; Weinberg, 1995). Current models indicate that Rb inhibits entry into S phase at least in part by binding E2F and inhibiting transcriptional activation. When cells sensitive to mitogen stimulation are treated with serum, Rb is phosphorylated, thus losing its ability to bind E2F and allowing activation of S-phase entry genes.

Mounting evidence suggests that there is a serum-responsive pathway dedicated to the regulation of Rb phosphorylation in which the kinase complex cyclin-dependent kinase (CDK) 4/6-cyclin D is the mitogen-responsive kinase of Rb (Sherr, 1994; Hunter and Pines, 1994). This kinase activity is regulated in part by the proteins of the INK4 class of cyclin-dependent kinase inhibitors (CDIs) (Sherr and Roberts, 1995), which specifically bind to and inhibit CDK4 and 6 (Serrano et al., 1993; Hannon and Beach, 1994; Sheaff and Roberts, 1995; Koh et al., 1995). p16^{INK4} can only inhibit entry to S phase or colony formation in cells that express Rb, while inhibition of cyclin D1 is effective in blocking S-phase entry only in cells containing Rb, thus establishing a functional link between CDK4 and Rb in cell cycle control (Guan et al., 1994; Medema et al., 1995; Lukas et al., 1994, 1995). In normal muscle, the CDIs p21 and the p16^{INK4}-related protein p18 are highly expressed (Parker et al., 1995; Guan et al., 1994), with p21 being induced by MyoD expression (Halevy et al., 1995; Guo et al., 1995). These high levels of CDIs presumably inhibit the mitogen-induced phosphorylation of Rb. In view of the plasticity of newt cells, a possible difference between urodele and other vertebrate cells may lie in the regulation of Rb function.

We are attempting to induce myotube dedifferentiation in cultured newt cells where it may be possible to analyze the underlying mechanism. This should provide insight into the process of regeneration and also shed light on how the differentiated state is regulated. Here we report that in contrast with those of other vertebrates, cultured newt myotubes formed from myogenic newt cells enter and traverse S phase when stimulated with serum. In comparison to the behavior of mammalian Rb^{-/-} myotubes (Schneider et al., 1994), an attractive explanation for the response of newt myotubes is that they lack Rb, but we show that these cells express Rb and that phosphorylation of Rb is an endpoint of the serum stimulation pathway. These results suggest that the regulation of Rb phosphorylation is likely to be a

key difference that distinguishes the behavior of newt and mammalian myotubes.

Materials and Methods

Cell Culture

Newt A1 cells were propagated as described (Ferretti and Brockes, 1988), myogenesis was induced, and myotubes were purified as described earlier (Lo et al., 1993). In brief, myotubes were formed by lowering the serum concentration from 10 to 0.5%, purified after 4 d by brief trypsinization, followed by neutralizing with media, sieving through a 100- μ m mesh (Cell MicroSieve; BioDesign Inc. of New York, Carmel, NY), and then sieving through a 35- μ m mesh. Myotubes were retained on this second sieve while mononucleate cells passed through, and then washed off the sieve into dishes precoated with 0.75% gelatin. For preparations requiring lowest contamination of mononucleates, the myotubes were sieved onto 35- μ m meshes twice before plating.

Bromodeoxyuridine Labeling and Immunofluorescence

To induce DNA synthesis, serum was raised to 10–20% at 1–2 d after purified myotubes were plated. After 4 d in serum, myotubes were labeled for 24 h with 1 μ l/ml 5-bromo-2 deoxyuridine/5-fluoro-2-deoxyuridine using the proliferation kit (Amersham Corp., Arlington Heights, IL) according to instructions. Cells were fixed for 5 min with methanol, stained for bromodeoxyuridine (BrdU) as previously described (Barres et al., 1994), and then stained for muscle-specific myosin using mAb against muscle-specific myosin heavy chain (A4.1025), a kind gift of Dr. Simon Hughes (Randall Institute, Kings College London, UK). FITC rabbit anti-mouse antibody was used as a secondary antibody, and all antibodies were diluted in 10% goat serum in PBS. For [³H]thymidine labeling, cultures were exposed to 1 μ Ci of [³H]thymidine per ml for 24 h, fixed with methanol, processed for antibody staining, refixed with methanol, and then coated with photographic emulsion (1:1, K-5; Ilford, Knutsford, Cheshire, UK) and developed after 2 d.

Determination of DNA Content

A more detailed account of the quantitative microscopy will be published elsewhere. In brief, cells were fixed with methanol, washed with PBS, treated with 2 N HCl for 5 min, washed with PBS, and stained with 10 μ M propidium iodide for 30 min. Propidium iodide was also included in the mounting medium. The laser-scanning microscope (Biorad MRC-500; Zeiss, Oberkochen, Germany) was used with a \times 5 objective, with the diaphragm fully open, and aligned for a maximally even field of illumination. Image analysis routines were used to threshold nuclei and to eliminate all background so that the intensity of individual nuclei could be measured in a semi-automated fashion. G₁-, G₂-, and S-phase peaks were determined by using mononucleate cells that had been labeled for 2 h with BrdU. Nuclei with no BrdU labeling clustered around two peaks: a large peak representing G₁ cells with 2N DNA, and a small peak, twice as intense as the first peak, representing G₂ cells with 4N DNA. BrdU⁺ nuclei displayed intensities between these peaks. 4N DNA was also confirmed by measuring the intensity of mitotic cells.

Growth Factor Assay

Myotubes were purified and plated in low serum (0.5%) media. Purified growth factors were added after 24 h, and cells were labeled 4 d later with BrdU for 24 h. Assays were performed in triplicate and at least 300 nuclei were counted per well. Mononucleate cells were plated at low density in low serum (1%) media for 24 h, before growth factor addition, and BrdU uptake was assayed 4 d later. Assays were performed in triplicate and at least 500 cells were counted per well. The growth factors were a kind gift of Dr. Mark Noble (Huntsman Cancer Institute, Salt Lake City, UT). Serum was delipidated as described (Rothblat et al., 1976).

Contact Inhibition Experiments

To form myotubes at varying densities, $\sim 1.3 \times 10^5$, 2×10^5 , or 4×10^5 A1 cells were plated onto 6-cm-diam tissue-culture dishes. After 24 h, myogenesis was induced by placing the cells in low serum media. After 5 d, serum was added to 20%, and then BrdU uptake and staining were assayed as described above.

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CDI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; CIP, calf intestinal phosphatase; GST, glutathione-S-transferase; Rb, retinoblastoma.

To vary the number of mononucleate cells, myotubes were purified in low serum media as described above and plated onto 3 × 3-cm-diam tissue-culture dishes. After 24 h, mononucleate A1 cells ranging in number from ~3 × 10⁴ to 26 × 10⁴ were plated onto the plates in low serum media. After 48 h, serum was added to 20%, and then BrdU uptake and staining were assayed as described above.

Tilt Experiment. Myotubes were purified in low serum media as described above and plated onto two 6-cm-diam tissue-culture dishes. After 24 h, ~2.5 × 10⁵ A1 cells were added, and the plates were tilted to allow cells to settle in a gradient of densities on the plate. After 48 h, serum was added to 20%, and then BrdU uptake and staining were assayed as described above.

Rb Cloning and RNase Protection

Degenerate oligonucleotides to the E1A binding region of Rb were used in a PCR reaction to obtain a 570-bp fragment from a λZAP retinoic acid-treated newt limb blastema cDNA library. This was used to probe a λgt11 limb blastema cDNA library (Brown and Brockes, 1991) from which two positive clones were isolated and sequenced. Both contained the 3' stop codon but lacked 600 bp of 5' sequence, which was obtained from the λZAP library by PCR using antisense oligonucleotides to sequences from within the gt11 clones together with λZAP primers. The amplified fragment was cloned in pBSK (Stratagene, La Jolla, CA). Total RNA was prepared and RNase protection was performed essentially as described (Brown and Brockes, 1991). Total RNA (10 μg) was annealed with a 330-bp probe from the E1A binding region to give the predicted 280-bp protected fragment. The gel was exposed for 2–3 d at –70°C.

Generation of Affinity-purified Polyclonal Antibody SK70

A glutathione-S-transferase (GST) fusion protein containing the newt Rb sequence from amino acids 269–447 was produced in bacteria, solubilized using sarcosyl (Frangioni and Neel, 1993), and purified using glutathione-Sepharose (Pharmacia, Uppsala, Sweden) followed by thrombin cleavage. Antibodies against the purified protein were produced in rabbits by Eurogentec (Seraing, Belgium). For affinity purification, IgG was precipitated from serum with ammonium sulfate and passed through a GST-myf-5 affinity column to remove anti-GST antibodies, then passed over an Affigel matrix coupled to the newt Rb fragment, and subsequently washed and eluted with glycine/HCl.

Immunoprecipitation and Western Blotting

Immunoprecipitation was performed essentially as described in Hu et al. (1991). Briefly, in experiments with mononucleate cells, ~800,000 cells were rinsed with PBS and lysed for 30 min on ice with 2 ml of lysis buffer. Cellular debris was removed by centrifugation for 1 min at 10,000 g. In initial experiments, extracts were precleared with rabbit serum and fixed *Staphylococcus aureus* as described (Harlow and Lane, 1988), but this was later found to be unnecessary. For immunoprecipitation with rabbit antibody, 1 ml of lysate was incubated with 1 μl SK70 on ice for 1 h, and then incubated 1 h with 10 μl protein A-Sepharose beads (Pharmacia). For immunoprecipitation with XZ56 (Hu et al., 1991), 1 ml of lysate was incubated for 2 h with 10 μl protein A-Sepharose beads that had been cross-linked with XZ56 antibody, using dimethyl pimelimidate as described in Harlow and Lane (1988). Phosphatase treatment of immunoprecipitates was essentially as described in Ludlow et al. (1989). Immunoprecipitated beads were washed twice with wash buffer (155 mM NaCl, 20 mM Tris, pH 8, 5 mM EDTA, 0.05% NP-40, 0.02% NaN₃), and then washed once with CIP buffer (100 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl₂). Beads were incubated with 40 U calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) per 60 μl buffer plus 2 mM PMSF, 20 μg/ml aprotinin, and 10 μg/ml leupeptin with or without phosphatase inhibitors (15 mM NaF, 150 μM sodium orthovanadate) for 15 min at 37°C, and then washed twice in wash buffer and solubilized in Laemmli sample buffer for 10 min at 80°C.

For detection of Rb in myotubes, myotubes were purified from 17 × 100-mm dishes plated onto two 100-mm dishes and kept in 0.5% serum (low serum) or 20% serum (high serum) for 4 d. One 100-mm plate of purified myotubes that contained ~50,000 myotube nuclei, with <10% mononucleate contamination, was lysed in 1 ml buffer and centrifuged as above. Samples were incubated with 1 μl SK70 for 1 h, then with 10 μl XZ56 for 1 h with rocking, and further with 10 μl protein A-Sepharose for

1 h. The samples were then washed three times with wash buffer before solubilization in Laemmli sample buffer for 10 min at 80°C. Samples were resolved on 7% SDS polyacrylamide gels, Western blotted onto nitrocellulose, blocked in 1% dried milk in TBS/0.1% Triton X-100 for 30 min, and then probed with SK70 at a 1:200 dilution followed by HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Corp.) and enhanced chemiluminescence (ECL; Amersham Corp.).

Δ34 and p16 Expression

The p16 expression plasmid (pcDNA3WTP16) was a kind gift of Drs. David Parry and Gordon Peters (Imperial Cancer Research Fund, London, UK). For p16 and control microinjections, plasmid (0.2, 0.6, or 1.0 μg/ml) was microinjected into myotube nuclei using an Eppendorf microinjection apparatus at 24 h after purified myotubes were plated in low serum media. Microinjection into a single nucleus per myotube was sufficient to generate expression of protein that spread throughout the cell cytoplasm. Myotubes were transferred to medium with 20% serum 24 h after injection, and BrdU uptake was assayed after 4 d as described above. The p16 protein was detected using mAb DCS-50.2 (a kind gift of Dr. Gordon Peters) at 2.6 μg/ml. In parallel control experiments, 0.2 or 1 mg/ml alkaline phosphatase expression plasmid (pCAP) (Schilthuis et al., 1993) was microinjected and BrdU uptake was assayed as above. Alkaline phosphatase was visualized with 5-bromo-4-chloro-3-indoyl-phosphate, and BrdU was detected using an HRP-conjugated secondary antibody.

The Δ34 expression plasmid was a kind gift of Dr. N. Jones (Imperial Cancer Research Fund, London, UK), and Rb plasmid pJ3WHRb was a kind gift of Dr. P. Jat (Ludwig Institute, London, UK). The DNA was microinjected at 0.5 mg/ml at 48 h after plating, and cells were placed in high serum media after an additional 48 h. [³H]Thymidine uptake was assayed as described above. This protocol, which uses longer times for plating and expression as compared with the p16 experiments, was adopted after the p16 experiments to optimize the number of myotubes obtained. Newt myotubes frequently require 48 h to attach fully, so this latter protocol allowed more cells to be injected. Rb protein expression was detected using mAb G3-245 (Pharmingen, San Diego, CA).

Results

Cultured Newt Myotubes Undergo S Phase in Response to Serum

In earlier studies it was shown that A1 newt limb cells stop dividing and fuse to form myotubes in low (0.5%) serum medium (Lo et al., 1993). The myotubes contract in response to mechanical stimulation and express markers of differentiation such as muscle-specific myosin heavy chain (Fig. 1 A). They could be purified from residual mononucleate cells using 35-μm cell sieves as described previously (Lo et al., 1993). Such preparations result in <10% contamination by mononucleates as assayed by counting nuclei. When purified myotubes were exposed to medium containing 10% FCS, they responded by synthesizing DNA as assayed by incorporation of BrdU. Fig. 1 shows two myotubes, both of which show positive immunostaining in their cytoplasm for muscle-specific myosin. Hoechst staining (Fig. 1 A) shows that each myotube contains multiple nuclei; those in the myotube on the right have incorporated BrdU (Fig. 1 B). In the preparation shown in Fig. 1, additional mononucleate cells were plated with the purified myotubes to illustrate that the BrdU incorporation in the myotubes is comparable to that in normal, proliferating cells and is unlikely to be due to the activation of DNA repair mechanisms.

The myotubes entered S phase somewhat asynchronously, starting 2 d after serum addition and with the highest number of nuclei incorporating BrdU at 4 d. Despite

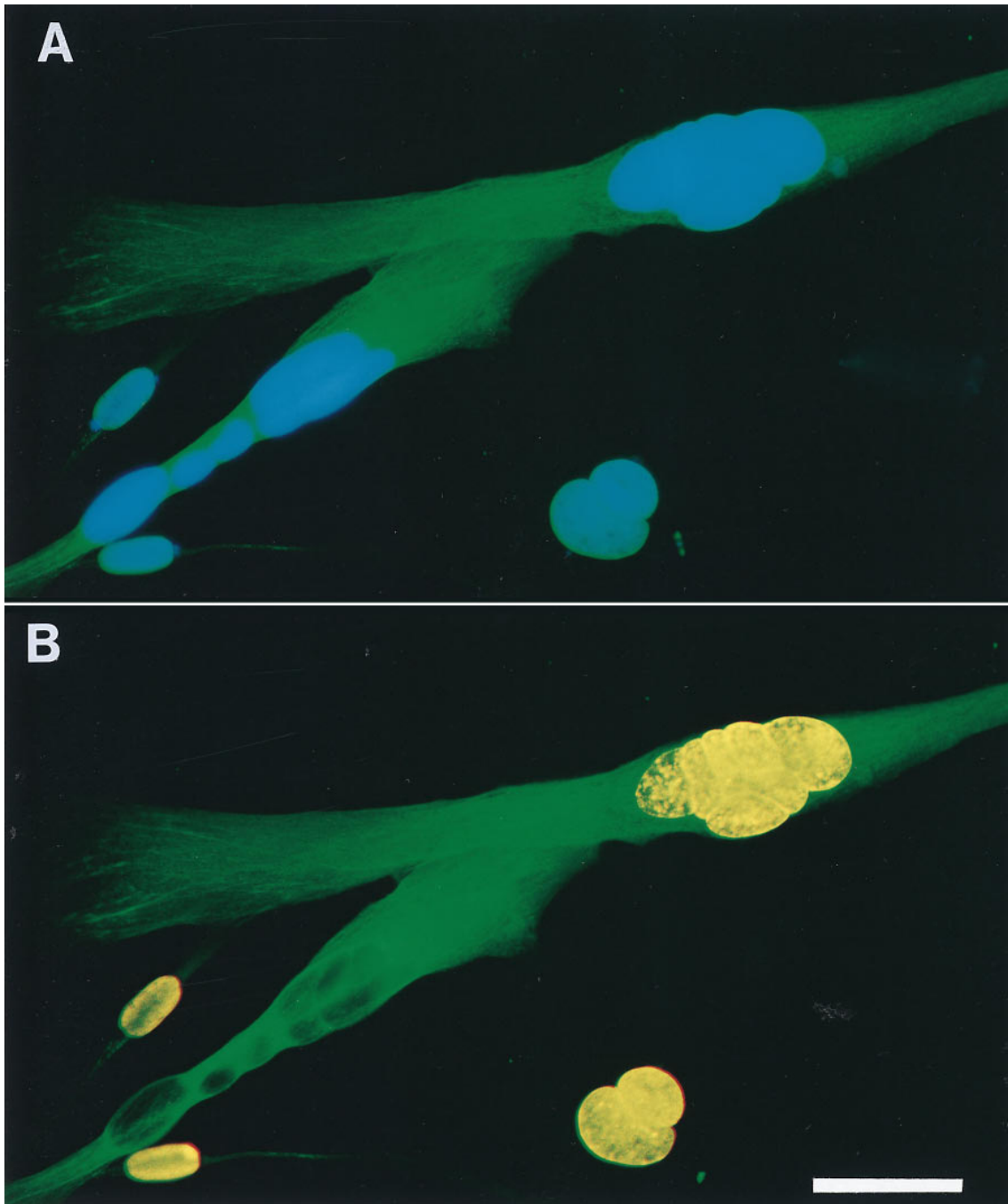
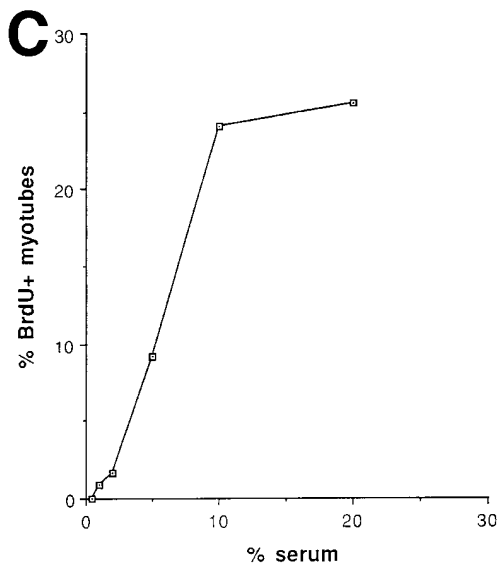


Figure 1. Serum stimulates newt myotubes to enter S phase. (A) The two A1 myotubes are stained with muscle-specific myosin (green) and Hoechst to stain DNA (blue). (B) The same myotubes showing muscle-specific myosin (green) and BrdU-positive nuclei (yellow). The myotube on the upper right has incorporated BrdU during a 24-h pulse given on day 4 after serum stimulation; the myotube on the lower left is negative for BrdU. (C, facing page) The graph shows the percentage of myotubes taking up BrdU at various concentrations of serum. Cells were labeled with BrdU for 24 h at 3 d after addition of serum. Bar, 100 μ m.

this delay, we found that serum stimulation for as little as 8 h was sufficient to generate the response. Furthermore, the uptake of BrdU within a myotube tended to include either all the nuclei or none at all. A dose response showed that the myotube response saturated at \sim 10% serum (Fig. 1 C). In this experiment, 25% of nuclei incorporated BrdU in a 24-h period on day 3, but overall at least 75% of the cells undergo DNA synthesis after stimulation. Such a response was not found in mouse myotubes. Myotubes formed from

the myoblast cell line, C2C12, were purified in a similar way, and BrdU incorporation was assayed up to 72 h after serum stimulation. Cell cycle reentry was not seen in these myotubes even after examination of several thousand nuclei.

To eliminate the possibility that the newt myotubes undergo an aberrant DNA synthesis or activate DNA repair, we determined if the nuclei traverse an S phase of normal duration by executing the protocol outlined in Fig. 2 A. Multiple plates of myotubes were labeled with an 8-h pulse



of [^3H]thymidine 2 d after serum addition, thus identifying those myotubes that had begun to synthesise DNA at this point. At varying times after the exposure to [^3H]thymidine, individual plates were labeled with BrdU for 24 h and then fixed and processed for immunohistochemistry and autoradiography. Nuclei that were [^3H]thymidine⁺ and BrdU⁺ were those that had started DNA synthesis on day 2 and were still undergoing DNA synthesis at the time of the BrdU labeling. Nuclei that were [^3H]thymidine⁺ but BrdU⁻ were those that had started DNA synthesis on day 2, but had completed it before the second labeling. As seen in Fig. 2 B, of the cells given the BrdU pulse immediately after the [^3H]thymidine, almost all [^3H]thymidine⁺ nuclei were also BrdU⁺, but of those labeled with BrdU 2 d after the [^3H]thymidine pulse (4 d after serum), the number of [^3H]thymidine⁺BrdU⁺ cells had decreased, and it was only 1% by 4 d after the [^3H]thymidine pulse (6 d after serum). This indicates that S phase lasts ~48–72 h in the myotube nuclei, which is similar to the length of S phase in mononucleate newt cells (Wallace and Maden, 1976). Significantly, this experiment also shows that the total number of [^3H]thymidine⁺ cells present remained relatively constant throughout the experiment, demonstrating that there was little cell death as a consequence of reentry.

There was no evidence of mitotic figures in the newt myotubes, even at 10 d after serum stimulation, suggesting that the nuclei arrest in G₂ phase. To confirm this finding, we determined the DNA content of myotube nuclei by fixing and staining the cells with propidium iodide and then measuring the fluorescence emission of the nuclei by scanning microscopy (see Materials and Methods). We validated the method by comparing the DNA content of serum-starved mononucleate cells where the majority of nuclei should be 2N (Fig. 3 A, dark bars, asterisk), with mononucleate cells grown in 10% serum where at least 50% of the cells should have DNA content between 2N and 4N (Fig. 3 A, hatched bars). The value for 4N DNA was confirmed by measuring the intensity of mitotic nuclei (double asterisk). Before fixation, the cells were labeled for 2 h with BrdU to determine the DNA content of the subset of cells that were in S phase or had just completed it (Fig. 3 A,

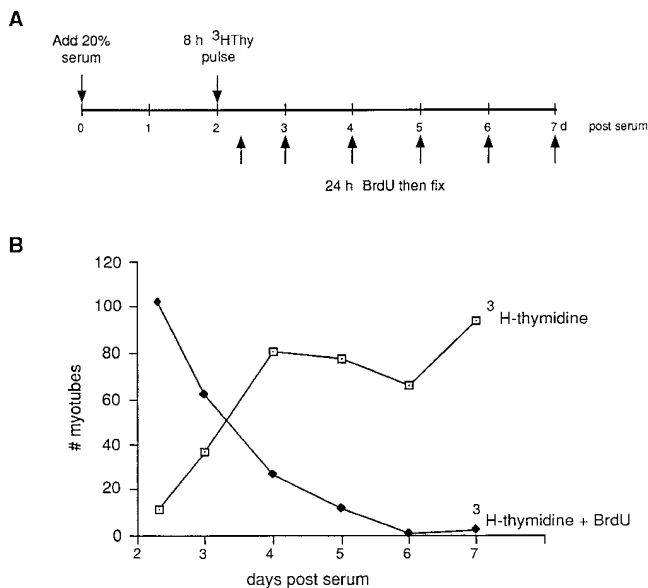


Figure 2. Myotube nuclei complete S phase in response to serum. (A) An outline of the experimental design to determine the duration of S phase in the myotubes (see text for details). Purified myotubes were labeled with an 8-h pulse of [^3H]thymidine 2 d after addition of 20% serum. At various times after this first pulse, samples were labeled with BrdU for 24 h (lower arrows) and fixed. (B) The number of myotubes positive for [^3H]thymidine is compared with those positive for both [^3H]thymidine and BrdU as the time between the [^3H]thymidine and BrdU pulses increases. The x axis denotes the time as shown in A. (Open squares) Number of myotubes containing only [^3H]thymidine-labeled nuclei. These cells had begun S phase at the time of the [^3H]thymidine pulse, but they had completed it before labeling with BrdU. (Closed diamonds) Number of myotubes containing nuclei positive for both [^3H]thymidine and BrdU. These cells had continued to synthesize DNA from the time of the [^3H]thymidine pulse to the time of the relevant BrdU pulse. Note that the majority of nuclei that had begun S phase on day 2 (when [^3H]thymidine was given) had completed it by day 5.

light bars). It is clear that the BrdU-positive nuclei have fluorescence emissions that lie between the 2N and 4N peaks. 8% of the serum-starved cells labeled with BrdU (data not shown).

We compared the distributions of fluorescence intensity of myotube nuclei maintained in low serum vs those that had been stimulated with serum for 9 d. To serve as an internal standard for 2N/4N peaks, mononucleate cells were seeded on the same plate as the myotubes in high serum media (Fig. 3 B). In low serum almost all myotube nuclei had intensities corresponding to 2N DNA (Fig. 3 B, dark bars), as would be expected for nuclei in G₀/G₁. In marked contrast, a significant number of nuclei in myotubes stimulated with serum had intensities around 4N (hatched bars). This confirms that the nuclei stimulated to undergo DNA synthesis were arrested in G₂.

Newt Myotubes Are Refractory to Several Common Polypeptide Growth Factors

Myoblasts divide in response to mitogens such as bFGF

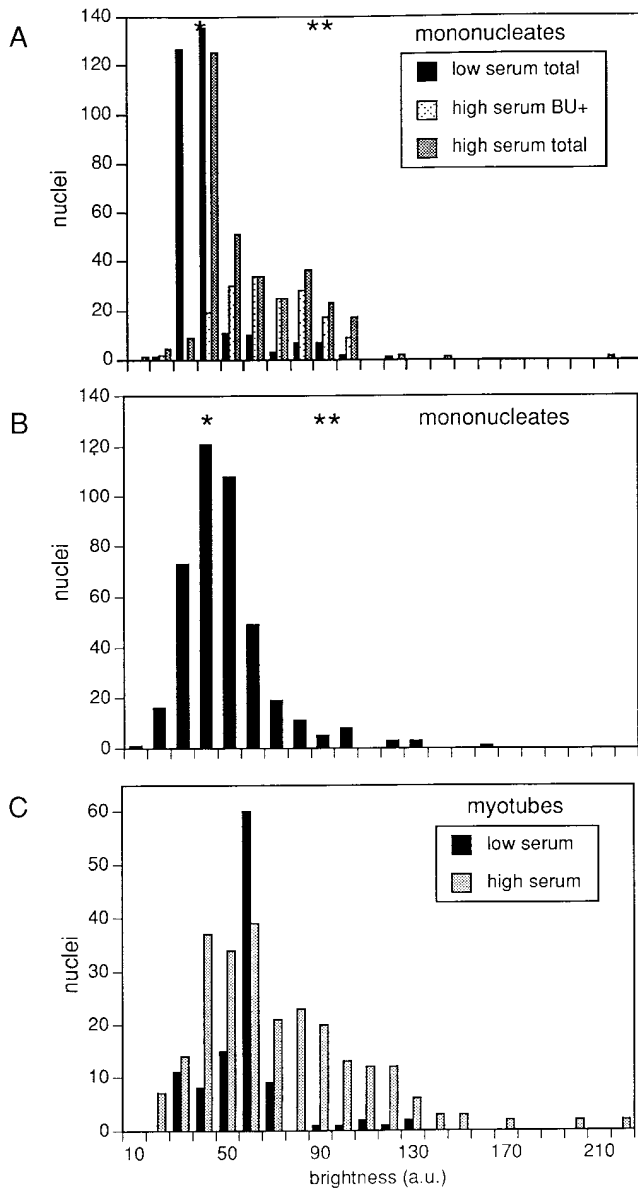


Figure 3. Nuclei with 4N DNA content accumulate in serum-stimulated myotubes. (A) A histogram of fluorescence intensity of propidium iodide-stained nuclei from mononucleate A1 cells. Intensity profiles from serum-starved cells (dark bars) and cycling cells maintained in high serum (hatched bars) were compared. (Asterisks) Intensities of 2N (*) and 4N (**) nuclei (see text and Materials and Methods). In addition, the profile generated by the subset of cells in high serum that had incorporated BrdU in a 2-h labeling is shown (light bars). Intensities fall between the 2N and 4N peaks. (B) The intensity profile of mononucleate cells plated with the myotubes in high serum shown in Fig. 3 C. These cells provided an internal standard to verify the intensities corresponding to 2N (*) and 4N DNA (**). (C) The DNA content of individual nuclei was measured in myotubes maintained in low serum vs those stimulated with high serum. (Black bars) DNA content of nuclei from myotubes kept in 0.5% serum medium for 9 d. Most nuclei have 2N DNA content, and very few have 4N, consistent with withdrawal from the cell cycle in G₁. (Hatched bars) DNA content of nuclei from myotubes kept in 10% serum medium for 9 d. A substantial proportion of cells have nuclei of 4N DNA content, consistent with passage through S phase and arrest before mitosis.

Table I. Effects of Serum and Purified Factors on Myotube Entry into S Phase

Substance	Myotubes	Mononucleates
bFGF	-	+
PDGF	-	+
EGF	-	+
IFG-1	-	+
TGFB	-	*
TNF α	-	ND
Delipidated serum	+	+
Dialyzed serum (MWCO 6,000-8,000)	+	+
Boiled serum	-	ND
Gel filtration (Superose 12)	150-300 kD	ND
Fibronectin	-	ND
Vitronectin	-	ND
Laminin	-	ND
Pretreatment of culture dish with FCS	-	ND

Growth factors were added at the following concentrations: 10 ng/ml bFGF, 10 ng/ml PDGF, 50 ng/ml EGF, 100 ng/ml IGF, 10 ng/ml TGFB, 200 and 2,000 U/ml TNF α . In assays of growth stimulation in mononucleate A1 cells, (+) represents a greater than fivefold increase in the number of BrdU⁺ cells as compared with cells growing in media containing 1% serum. In assays on myotubes, (-) represents <0.2% BrdU⁺ nuclei in an assay where 10% serum induced 30% BrdU⁺ nuclei. (*) TGFB induced growth arrest in mononucleate cells.

and EGF, and thus it was important to determine if the return of newt myotubes to the cell cycle was due to the retention of sensitivity to such growth factors. A number of polypeptide growth factors including PDGF, EGF, IGF, and FGF were tested (Table I), and although they each stimulated division of mononucleate newt A1 cells, none elicited cell cycle reentry of the newt myotubes. These factors were also assayed in the presence of 5% bovine plasma and no response was observed. It is thus apparent that the urodele cells are as refractory to these growth factors as their mammalian counterparts (Olson, 1992).

Cell Cycle Reentry Is Dependent on Cell Density

Unpurified newt myotubes formed from dense cultures of myoblasts did not consistently respond to serum. This suggested that cell cycle reentry is inhibited by contact with other cells, and this possibility was further investigated. First, we examined the response of myotubes formed from varying densities (ranging threefold) of myoblasts. Without purification, myotubes formed in dense cultures of myoblasts were unresponsive to serum, whereas those formed in sparse cultures did respond (Table II A). To determine if purified myotubes could also be regulated by cell density, we examined the effect of adding back A1 cells to purified myotubes. 24 h after plating purified newt myotubes, mononucleate cells were added at varying densities and cultured in low serum for 48 h before addition of serum. Myotubes surrounded by a subconfluent population of mononucleate cells responded to serum as well as purified myotubes, while myotubes surrounded by a confluent population of cells were inhibited from cell cycle reentry (Table II B). This inhibition of BrdU uptake into myotubes is significantly greater than the effect of density on the mononucleate A1 cells that appear to be relatively insensitive to contact inhibition. To determine if inhibition reflected direct cell-cell contact rather than the action of a soluble factor released by the mononucleate cells, we per-

Table II. Cell Cycle Reentry Is Sensitive to Cell Density

	High Density		Medium Density		Low Density	
	Myotube	Mononucleate	Myotube	Mononucleate	Myotube	Mononucleate
A. Myotubes fused at different densities	1/1,273 {0.1%}	91/290 {31%}	6/1,352 {0.4%}	124/302 {41%}	354/1,284 {28%}	126/276 {46%}
B. Added mononucleates	2/804 {0.2%}	89/485 {18%}	96/546 {18%}	148/300 {49%}	102/501 {20%}	
C. Tilt	1/1,527 {0.06%}	ND			56/606 {9%}	ND

Myotubes cultured with varying numbers of mononucleate cells show varying response to serum. (A) Unpurified myotubes formed from varying densities of myoblasts. High density represented starting cell densities of 140 cells per mm²; medium density represented 70 cells per mm²; and low density represented 46 cells per mm². (B) Varying numbers of mononucleate cells were plated onto plates of myotubes. Numbers represent the number of BrdU⁺ nuclei per total number of nuclei in myotubes or the mononucleate cells. High density corresponded to ~370 cells per mm²; medium density corresponded to 110 cells per mm²; and low density corresponded to no added cells. (C) Mononucleate cells were plated in a density gradient across a single plate of uniformly distributed myotubes. High and low density represent the counts for nuclei in myotubes in two extreme sectors of the plate, which were each 10% of the area of the plate.

formed the following experiment. After plating purified myotubes in a uniform distribution, mononucleate cells were attached in a gradient of density across the plate by tilting it. In the region of the plate where mononucleate cells were sparse, myotubes responded to serum, whereas, on the opposite side of the plate where cells were dense, myotubes were completely inhibited from responding (Table II C). This result provides strong evidence against a diffusible inhibitor and in favor of regulation by local cell contact.

Newt Myotubes Express Rb

As a result of the similarity of newt myotubes and murine Rb^{-/-} myotubes in their response to serum (Schneider et al., 1994), we determined if Rb or an Rb-like gene is ex-

pressed in the urodele cells. Full-length cDNA of newt Rb was isolated and sequenced as described in Materials and Methods. It encodes a protein with a predicted molecular mass of 103 kD (see Fig. 5) that is 62% identical to mouse Rb (Bernards et al., 1989) and 59% identical to *Xenopus* (Destree et al., 1992). It is interesting that the predicted newt protein, like *Xenopus* Rb, lacks the proline-rich sequences found at the NH₂ terminus of the mouse sequence.

RNase protection analysis revealed that Rb is transcribed in all of the tissues assayed, including A1 myoblasts and myotubes as well as normal limb and blastemal tissue (Fig. 4), and analysis by Northern blot revealed two transcripts of ~4 and 6 kb in normal limb tissue (not shown). As discussed further below, immunological detection of Rb indicated that the protein is expressed in the

A

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NEWT  MPPKYP.....PQVRKGDSSSSAVQSQBEHRQWLD....DAD
MOUSE  ---A-RRAAAAEPFPPP-PPPRE-DPAQDSQPE-LPLAR-EFEEIEBPE

NEWT  FTLLCDSLKVSADVREGSKWIKQVSDGCG...ERIRNKEVLGICIFLA
MOUSE  -IA-QK---P-H---RA-LT-E---SVDQILEGY-QKKKEIA----I-

NEWT  AVELADLFTITELLKSTHIVNMTYIKEMDSMDTITTRVNAVTRL
MOUSE  -D-DRMP-F---Q---IETS-YK-FDLL--I-?.....D--MS--

NEWT  KFKYNTLCTLYQKQPLPELLFIKPEPSNVQISSDISPAALFKILNITFLL
MOUSE  LK---V-A-S-LE-TC---YLTQ--S.AL-TE-NSMLVL--S-----

NEWT  VKGSLQMEDDLVISFOLLQCFVTHYIKILPLPLLEPFYKSAISGKSDKA
MOUSE  A--E-----M--VDYF--FS-PA-----T..AIPING

NEWT  QTRSSRGRNRNPHLSKQVESDE..VFILCKENGCNMDEVKNINITFPVP
MOUSE  SP--TP--Q--SARIA-L-N-TRII-V---HE--I-----VYFKN-I-

NEWT  FLDSVGISTNGIFREPVLKQYEDLIFNSKDLARFLDDEDTLQPNNG
MOUSE  -IN-L--VSS--L--VES---R--EV-LKN-----H-K---TDPFI

NEWT  GCVELEKTPQKNDP..EEMNLIPQTPVPTAMNTIQNLNLSLASDQSPSP
MOUSE  DSP-T-R-R--N-D--A-VVT-H-----V---Q-MVI-N-----E

NEWT  TLKAYFNNTSNPTRAIQERENNLQIFKQFAEAVQACEEIGYQRFKV
MOUSE  N-IS-----V--EEN-LK-VKDV-H--E--N--G-VD--V--Y-L

NEWT  GVRLAYRVMEAMLSSEKRLSQVNSFLNNSAPHESSLACSEVVMATY
MOUSE  ---Y---S-----E--I--K--DNI--M-----AL-----

NEWT  GS...ISRETDLSFFWILLEVFKIEYDYPYKVIKESFKDEPFLREMIK
MOUSE  SRSTLQLHDSG-----N-LNLKAF-----V-AN-----

NEWT  HLESCHEHRIMESLAWRSESPYIELTOPRREGHVQPEPTSSLMQPLEH
MOUSE  ---R-----L-D-LFD--K-SKDG--P--NL--ACP-SL--QG

NEWT  NHTAADLYLSPLRSPRRNVPTSRALPTLFSVESNNVPALPQPTQRSTSL
MOUSE  ---M-----KKRTS-T..VNSAANT-TQAS-PHT-KPLK--

NEWT  SLEFYKRVYRLAYLRMNTLCKLDDPPELHVITLQHTLQNEVELMKD
MOUSE  A-----L--AR--S-----I-----R-

NEWT  RHLQQLMCSWYAIKVKVAILDAFKTIVTAYKELPNTQPTFKHWLIRDG
MOUSE  -----G-----N--K--I-----D--HAA-----R---ER

NEWT  QHDSIIIVFYNLVMQKLTNQLYATLRNPTLSPHPIPRSPYKISNSPL
MOUSE  EF-----S-----R-----ST-P-----F-S---

NEWT  RLPQGNIIYISPLKSPYKHEGLLSPTRKMTPRSRILVIGQFGTAEKFKQ
MOUSE  -I-----IS-----PT-----S-----S-----

NEWT  KINQMLSSSERLKRRAESNSAPKLRKLRHFDTPDQPDGSKHLA...
MOUSE  ---VCN-D-VL--S--GG-P-----NVR--IE-A-A-----PAESK

NEWT  ..QKLAEMTSTRSMQKQKQDEMDTTLKEEK
MOUSE  FQ-----T-----RMM-SK-VSN---
```

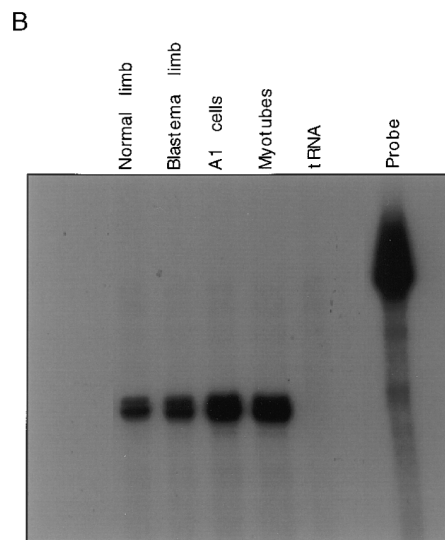


Figure 4. Sequence and expression of newt Rb. (A) The amino acid sequence of the newt Rb protein, predicted from the cDNA sequence, as compared with that of mouse. (-) Positions where identical residues are found. (.) Positions at which gaps have been introduced to optimize the alignment. As with *Xenopus* Rb, newt Rb lacks the distinct NH₂-terminal proline-rich sequence found in the mouse sequence. The predicted size of the encoded protein is 103 kD, compared with 105 kD for mouse and 103 kD for *Xenopus*. The overall identity with mouse is 62%, and with *Xenopus* 59%. These sequence data are available under the EMBL/GenBank/DBJ accession number Y09226. (B) RNase protection assay demonstrating that newt Rb is expressed in the A1 cells and myotubes, as well as in normal limb and limb blastema tissue.

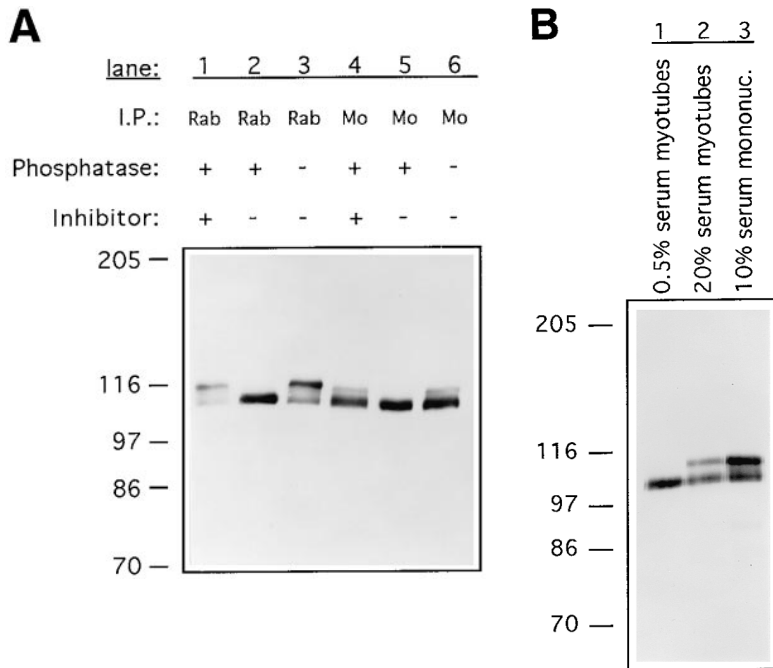


Figure 5. (A) The SK70 antibody recognizes phosphorylated Rb. Affinity-purified polyclonal rabbit antibody (SK70) prepared against newt Rb was used to immunoprecipitate and detect Rb by Western blotting from mononucleate A1 extracts (lanes 1–3). Immunoprecipitates were either treated with calf intestinal phosphatase (CIP) plus phosphatase inhibitors, NaF and orthovanadate (lane 1); treated with CIP with no inhibitors (lane 2); or directly prepared for electrophoresis without any phosphatase treatment (lane 3). Separate extracts were immunoprecipitated with XZ56, an Ab against human Rb, and probed on the same Western blot using SK70 (lanes 4–6). Immunoprecipitates were either treated with CIP plus phosphatase inhibitors (lane 4) or CIP with no inhibitors (lane 5) or directly prepared for electrophoresis (lane 6). The phosphatase-treated immunoprecipitated protein (lane 5) migrates at precisely the same molecular weight as the phosphatase-treated protein that is immunoprecipitated by SK70 (lane 2). (B) Profile of Rb phosphorylation in purified myotubes maintained in low vs high serum. Extracts made from highly purified myotubes maintained in low (lane 1) and high serum (lane 2) media and from mononucleate cells (lane 3) were

immunoprecipitated simultaneously with SK70 and XZ56 to detect all forms of Rb and Western blotted using SK70. Myotubes in low serum media have withdrawn from the cell cycle and contain only the nonphosphorylated form of Rb (lane 1). Myotubes stimulated with high serum media to reenter the cell cycle contain phosphorylated forms of Rb (lane 2). This profile is similar to the profile of Rb migration found in proliferating mononucleate cells (lane 3).

myotubes (Fig. 5 B). It is clear, therefore, that the myotubes are not Rb negative.

Phosphorylation of Rb Is Required for Cell Cycle Reentry

It was important to determine if cell cycle reentry of the newt myotubes occurred via the phosphorylation of Rb. To this end, we first asked whether expression of the CDI, p16, which specifically inhibits the CDK4/6 class of kinases, is able to inhibit the serum response. This would implicate Rb phosphorylation in cell cycle reentry, and it would indicate that the phosphorylation of Rb occurs via activation of CDK4/6. When human p16 was expressed in the newt myotubes, it completely inhibited uptake of BrdU (Table III). Control injections with plasmids expressing alkaline phosphatase had normal levels of DNA synthesis.

To test more directly whether phosphorylation of Rb was required for the serum response, we determined if expression of the $\Delta 34$ Rb mutant (Hamel et al., 1992), in which all eight of the CDK consensus phosphorylation sites have been mutated, blocked entry into S phase. This mutant should compete with endogenous Rb and therefore inhibit any events that require Rb phosphorylation. Myotubes maintained in low serum media were transfected by microinjection with a plasmid encoding either the $\Delta 34$ Rb or wild-type Rb as a control. 2 d after microinjection, myotubes were transferred to high serum, and, after 4 d, myotubes were assayed for [3 H]thymidine incorporation, with Rb expression detected by immunofluorescence using mAb G3-245. As seen in Table IV, expression of $\Delta 34$ Rb resulted in 88% inhibition of DNA synthesis as compared with only 30% inhibition by wild-type Rb.

The Phosphorylation of Newt Rb during Serum Stimulation

We next examined the phosphorylation state of endogenous Rb in the newt myotubes. In mammalian cells such as quiescent fibroblasts and resting lymphocytes, Rb is hypophosphorylated and becomes phosphorylated when the cells are stimulated to enter S phase. This transition can be detected by a mobility shift on SDS gels. When an affinity-

Table III. Expression of Human p16 in Myotubes Inhibits Reentry to the Cell Cycle

	BrdU ⁺ nuclei Total nuclei	Percentage
		%
p16	0	0
Uninjected	506 252 771	33
Alkaline phosphatase	78 158	49
Uninjected	271 572	47

Separate plates of myotubes were microinjected with an expression plasmid encoding either the CDK inhibitor p16 or, as a control, human placental alkaline phosphatase. The myotubes were stimulated with serum, and entry into S phase was measured as described. The top panel shows the proportion of nuclei in p16-positive myotubes that took up BrdU compared with the equivalent proportion for uninjected myotubes on the same plates. The bottom panel shows the equivalent proportions for the nuclei in alkaline phosphatase-positive myotubes compared with uninjected control myotubes on the same plates. A block of entry into S phase by p16 is evident, whereas the alkaline phosphatase control has no effect. This shows the results of a single experiment in which DNAs were injected at 0.2 mg/ml. In other experiments, p16 had a similar effect when injected at 0.6 or 1 mg/ml, whereas alkaline phosphatase had no effect at 1 mg/ml.

Table IV. Expression of $\Delta 34$ Rb Inhibits Cell Cycle Reentry

Rb type	Percentage of inhibition	n	Number of injected nuclei
$\Delta 34$	88	4	612
Wild-type	30	4	1,447

Plates of myotubes were microinjected with either $\Delta 34$ Rb or wild-type Rb as a control. The myotubes were stimulated with serum, and entry into S phase, as assayed by [3 H]thymidine, was measured as described. To calculate percentage of inhibition, the fraction of Rb-immunopositive cells that were positive for [3 H]thymidine was counted (denoted a), and the fraction of nuclei in uninjected myotubes on the same plate that were positive for [3 H]thymidine was counted separately (denoted b). Percentage of inhibition = $100 - (100 \times a/b)$. For each plate, at least 1,000 nuclei from uninjected myotubes were counted.

n, number of experiments; Number of injected nuclei, total number of Rb-immunopositive nuclei counted in all four experiments.

purified polyclonal rabbit antibody (SK70) prepared against a portion of the pocket region of newt Rb was used to analyze Rb in extracts of proliferating mononucleate A1 cells by immunoprecipitation and subsequent Western blotting, a series of bands spanning 110–116 kD was specifically recognized (Fig. 5 A, lane 3). Treatment of the immunoprecipitate with calf intestinal phosphatase caused the protein to migrate as a single 110-kD band (Fig. 5 A, lane 2), a change blocked by inclusion of phosphatase inhibitors (Fig. 5 A, lane 1). The identity of these bands as Rb was further confirmed by immunoprecipitating extracts of mononucleate cells with an mAb against human Rb (XZ56; Hu et al., 1991), which is known to cross-react with *Xenopus* Rb, and then by Western blotting with SK70 (Fig. 5 A, lane 6). In this case, a doublet at 110 kD was recognized. Phosphatase treatment of the immunoprecipitate resulted in migration of the protein at the same molecular mass as the phosphatase-treated protein from the SK70 immunoprecipitate (Fig. 5 A, lanes 5 and 2, respectively). Based on mobility, the SK70 antibody under immunoprecipitation conditions appears to preferentially recognize more highly phosphorylated forms of newt Rb as compared with XZ56.

To compare the phosphorylation state of Rb in myotubes maintained in low serum vs high serum, extracts from highly purified myotubes (where the mononucleate contamination was <10%) were immunoprecipitated with both SK70 and XZ56, to obtain all forms of Rb, and then detected by Western blotting with SK70. The Rb that was immunoprecipitated from extracts of myotubes maintained in low serum migrated as a single band at 110 kD, indicating that none of the Rb was phosphorylated (Fig. 5 B, lane 1). In contrast, ~50% of Rb from myotubes maintained in high serum for 4 d had retarded mobility (Fig. 5 B, lane 2). At this time, 25–50% of myotubes are expected to be in S phase. This profile was identical to the profile of Rb that was immunoprecipitated from proliferating mononucleate cells on which the antibodies were characterized (Fig. 5 B, lane 3), and, as with the mononucleate cells, phosphatase treatment of the immunoprecipitate caused the Rb to migrate as a single band (data not shown). Detection of rabbit IgG confirmed that equivalent amounts of SK70 were immunoprecipitated in each of the samples (data not shown). Thus, like other vertebrate cells that undergo a transition from the G₀ or resting state to a proliferating state, the Rb in myotubes maintained in low serum is

solely in the nonphosphorylated form but becomes phosphorylated after stimulation by serum to enter S phase.

Discussion

Urodele amphibians have the remarkable ability among adult vertebrates to regenerate much of the body plan including the limbs, tail, and jaws, as well as ocular tissues such as the lens. Regeneration proceeds by the local reversal of differentiation of adult tissues to provide the proliferating mesenchymal or epithelial progenitor cells of the regenerate (Brockes, 1994; Okada, 1991; Wallace, 1981). After removal of the lens, pigmented epithelial cells of the dorsal iris lose their pigment and proliferate before transdifferentiating into lens, a transition that can be reproduced in clonal cell culture (Eguchi et al., 1974; Okada, 1991). In limb regeneration, the blastemal progenitor cells arise by dedifferentiation of mesenchymal tissues at the amputation plane (Hay, 1959; Steen, 1968; Namenwirth, 1974; Lo et al., 1993). It is unclear to what extent urodele cells are intrinsically different from those of other vertebrates, and to what extent they encounter distinct signals that evoke reversal.

Here we have shown that newt myotubes are different from their mammalian counterparts. In culture, newt limb cells withdraw from the cell cycle in low serum medium and form myotubes, which are refractory to growth factors, but respond to elevated serum by entering S phase. This response is striking because although it is known that nuclei within mammalian myotubes are capable of synthesizing DNA in response to the expression of certain viral oncogenes (Endo and Nadal-Ginard, 1989; Iujvidin et al., 1990; Crescenzi et al., 1995), deletion of the Rb gene (Schneider et al., 1994), or infection by the parasitic nematode *Trichinella spiralis* (Jasmer, 1993), this is the first demonstration in which multinucleate myofibers undergo S phase in response to serum stimulation without the need for direct manipulation of internal cellular components. Furthermore, it is significant that not only do these newt myotubes enter S phase in response to serum, they do so without apparent trauma or evoking apoptosis. This is in contrast with the degeneration seen when SV-40 large T induces reentry in mammalian myotubes (Endo and Nadal-Ginard, 1989; Iujvidin et al., 1990).

It seems likely that the reentry response of newt myotubes underlies their ability to dedifferentiate during regeneration. Histological examination of muscle fibers in early regenerating limbs labeled with [3 H]thymidine has identified some fibers containing labeled nuclei (Hay, 1959). Although these experiments could not definitively distinguish between cells that had newly fused to form fibers as opposed to nuclei returning to the cell cycle within fibers, they are consistent with entry into S phase being a very early step in endogenous dedifferentiation.

If induction of S phase is the initial step in muscle dedifferentiation during limb regeneration, then, in vivo, these muscle cells must go on to resolve into mononucleate cells. Indeed, Lo et al. (1993) showed that the same myotubes as those used here do form mononucleate cells when implanted into the regenerating limb. The execution of mitosis with attendant cytokinesis seems a likely means by which the nuclei in the syncytial myotube could resolve

into individual cells, although other mechanisms that do not rely on reentering the cell cycle are possible. It is most likely that in culture an additional signal is required to overcome the G₂-M arrest, and this is not present in FCS. It is also possible that a separate signal inducing further reversal of differentiation is required before the G₂-M block can be relieved. A third possibility is that the configuration that the myotubes assume in culture, in which the nuclei cluster in the center rather than disperse along the fiber, is not conducive to resolution into mononucleate cells.

What is the molecular basis of plasticity in the newt myotubes? Experiments in mammalian myotubes where elimination of Rb function, either by genomic deletion or inactivation by viral proteins, results in S-phase entry by myotube nuclei indicate that differences in regulation of Rb may underlie the ability of newt myotubes to retain a serum response after differentiation. In normal cycling cells, phosphorylation of Rb appears to be the predominant means of inactivating Rb function at the G₁ to S transition. In differentiated cells such as mammalian muscle, cell cycle arrest is not relieved by serum stimulation presumably because the kinases such as CDK4 that normally phosphorylate Rb in response to mitogens are inhibited from doing so by CDIs present at high levels in these cells (Guan et al., 1994; Parker et al., 1995). In Rb^{-/-} mouse myoblasts, the Rb-like protein p107 appears to substitute for Rb to allow exit from the cell cycle and differentiation, but, unlike Rb, expression of p107 in the myotubes is downregulated in response to serum, thus bypassing the need for phosphorylation (Schneider et al., 1994). Alternatively, when viral oncogenes are expressed in a myotube, they bind and sequester Rb (Gu et al., 1993), thus driving the cells into S phase.

The situation in the newt cells is rather different. We have shown that newt myotubes express Rb, which is phosphorylated upon cell cycle reentry. Furthermore, expression of the CDI p16 as well as expression of a mutant Rb lacking all CDK consensus phosphorylation sites inhibits the serum response, indicating that phosphorylation of Rb is a critical step in cell cycle reentry in these cells. The dramatic inhibitory effect of p16 expression is consistent with previous work indicating that high levels of p16 effectively inhibit the ability of cyclin D to bind cdk4 and cdk6 both in vivo and in vitro (Xiong et al., 1993; Parry et al., 1995). In this case, the complete inhibition of kinase should result in none of the endogenous Rb becoming phosphorylated by CDK4/6. In contrast, when the Δ34 Rb mutant is expressed, the endogenous Rb is presumably still subjected to mitogen-stimulated phosphorylation. This competition between the mutant and the endogenous Rb for binding to factors such as E2F may explain the incomplete inhibition seen in the Δ34 Rb expression experiments.

It appears that in newt myotubes serum stimulation induces S phase through activation of a cdk4/6-cyclin D or related kinase that phosphorylates Rb. The simplest model would posit that p16 or other CDIs are either not produced in the urodele cells, or are downregulated or inactivated by the serum pathway. Of course, we do not know if the effect is direct, or if other factors might be involved between serum stimulation and Rb phosphorylation. The newt myotubes illustrate nonetheless that irreversible in-

activation of the Rb phosphorylation pathway is not a prerequisite of muscle differentiation.

It will be of interest to identify the extracellular factors that regulate S-phase entry in the newt myotubes. The present results distinguish two classes of factors—a stimulatory soluble activity that is present in serum, and a dominant inhibitory factor on the surface of other cells that results in contact inhibition. These two contrasting activities may explain how specificity of the response is achieved in vivo. In general, newt muscle must be as firmly locked in the differentiated state as its mammalian counterpart, with dedifferentiation only being triggered under the special circumstances of amputation. In this context, it is perhaps not surprising that the reentry response is not stimulated by standard growth factors and that this aspect of the post-mitotic arrest is intact. It is an attractive hypothesis that amputation relieves the inhibition of cell-cell contact, thus allowing muscle fibers and other differentiated cells at the amputation plane to respond locally to soluble serum factors.

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