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# Satellite Cell Proliferative Compartments in Growing Skeletal Muscles

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The cell cycle time of satellite cells in growing rats was determined to be approximately 32 hr, with an S-phase of 14 hr. The estimated cycle time was the same for satellite cells in both oxidative soleus and glycolytic EDL muscles and is consistent with the rate at which myonuclei are produced during growth. Continuous infusion of bromodeoxyuridine (BrdU) was used to determine if all satellite cells had the same cycle time *in vivo*. Approximately 80% of the satellite cell population was readily labeled over the first 5 days of continuous infusion. Remaining satellite cells accumulated label at a much slower rate and were still not completely saturated after an additional 9 days of infusion. Only a small portion of the cells labeled with BrdU during the first 5 days could be labeled with a second label ([$^3$H]thymidine) during tandem continuous infusion experiments, suggesting that they pass through a limited number of mitotic divisions prior to fusion. These results suggest that satellite cells in growing oxidative and glycolytic skeletal muscles can be subdivided into two distinct compartments. About 80% divide with a 32-hr cell cycle duration and are responsible chiefly for providing myonuclei to growing fibers. The remaining 20% of the cells divide more slowly, probably because the cells enter a $G_0$-phase between mitotic divisions. These reserve cells, through asymmetric divisions, may generate the myonuclei-producing satellite cell population. Proliferative potential for regeneration and adaptive responses is likely located in this reserve population. 

## INTRODUCTION

Skeletal muscle satellite cells (Mauro, 1961) in immature growing muscle are responsible for providing additional myonuclei to enlarging myofibers. Myonuclear accretion is accomplished by continued mitotic divisions of this population throughout the growth period and by fusion of a portion of the daughter cells produced by the mitoses. Moss and Leblond (1971) clearly demonstrated that immediately after a single pulse injection of [$^3$H]thymidine only satellite cell nuclei were labeled on myofibers. However, as the interval between the injection and time of assay was lengthened, both satellite cells and myonuclei were labeled. They concluded that satellite cells in immature muscles constitute a dividing cell population and function to provide additional myonuclei by fusing with their myofibers. Due mainly to a lack of evidence to the contrary, it is generally accepted that each cell participates equally in the production of myonuclei.

The number of myonuclei generated during an interval of the growth period is dependent not only upon the number of satellite cells completing terminal divisions, but also upon the number of divisions possible by each cell within a given period, with the latter determined by the duration of the cell cycle. For example, approximately 93 satellite cells (based upon an estimate of 12% satellite cell nuclei) are available on each tibialis anterior myofiber, to provide the 20 myonuclei/day required at the 15-day-old growth rate (Enesco and Puddy, 1964; Moss and Leblond, 1971). If all satellite cells on a myofiber were cycling with a 12-hr cell cycle duration (Bischoff, 1986,1989, 1990), and 50% of the daughter cells fused with the fiber (Moss and Leblond, 1971), there would be a significant overproduction of myonuclei. Therefore, the estimated myonuclear accretion during the specific growth period examined by Enesco and Puddy (1964) is more consistent with (1) a cell cycle time that is greater than *in vitro* estimates, (2) only a subpopulation of satellite cell population providing myonuclei to the fibers, (3) only a fraction of divisions producing satellite cells available for fusion, or (4) some combination of these.

*In vitro* studies provide the basis for the hypothesis that satellite cells *in vivo* are not a homogeneous population. When plated and grown at clonal density, colonies derived from single satellite cells exhibit a wide range of sizes (Schultz and Lipton, 1982). The majority of cells from rat muscle form small colonies, whereas a minority form relatively large colonies. This colony size diversity suggests that satellite cells in a muscle may have different fates *in*

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*vivo* and, therefore, may not share equally in the formation of myonuclei. For example, some mitotic divisions may not produce progeny that are immediately available for fusion, but rather daughter cells that remain in the dividing population. In this sense, some cells function as reserve cells. Limited mitotic divisions by reserve satellite cells would conserve proliferative capacity during growth for regeneration and repair. Alternatively, small colonies derived from some satellite cells *in vitro* may reflect those cells programmed to fuse after only a few divisions and consequently, cells more intimately involved in the production of myonuclei (Grounds and McGeachie, 1987). Moss and Leblond (1971) found that approximately 50% of labeled daughter cells fused with a myofiber 48 hr after a single injection.

In this study, the cell cycle time of satellite cells in growing rat soleus and EDL muscles was measured *in vivo,* and their division behavior was monitored to obtain a better understanding of the dynamics involved in producing myonuclei during a specific growth period and the manner in which a proliferative reserve is maintained for regeneration and repair. We find that satellite cell behavior *in vivo* is distinctly different than that seen *in vitro* in terms of the number of cells that are dividing and available for fusion and in terms of the duration of the cell cycle.

## MATERIALS AND METHODS

A total of 78 male, 30-day-old Sprague–Dawley rats were used in this study. All procedures were approved by the University of Wisconsin Animal Care Committee.

### *Cell Cycle Parameters of Satellite Cells in Vivo*

*Duration of S-phase.* The duration of S-phase was determined by the double label method (Baserga and Nemeroff, 1962; Wimber and Quastler, 1963). The two markers of DNA synthesis were [$^3$H]-thymidine, administered at a dosage of 2 $\mu$Ci/g body weight, and 5-bromo-2-deoxyuridine (BrdU), at a dosage of 10 $\mu$g/g body weight. All injections of label were intraperitoneal and [$^3$H]thymidine was always injected first. Animals were killed 1 hr after the second injection. The intervals between the first and second injection were 6 ($n$ = 6) and 7 hr ($n$ = 6). Extensor digitorum longus (EDL) and soleus muscles were fixed in Carnoy's fixative and processed to obtain individual myofibers segments according to the process of Kopriwa and Moss (1971), which is a process that ensures that only labeled satellite cell nuclei are examined (Schultz, 1979). Single fibers isolated using collagenase digestion were immunostained for BrdU. Primary antibody was mouse anti-BrdU (1:15 dilution; Becton Dickinson) followed by GAM–peroxidase (1:50 dilution; Hyclone). After the fibers were treated with diaminobenzidine they were washed and suspended in NTB2 photographic emulsion (Kodak) and exposed at 4°C for periods of up to 4 weeks. The slides were developed and the number of labeled nuclei was tabulated as singly labeled with either [$^3$H]thymidine or BrdU or doubly labeled. Unlabeled nuclei were ignored. The duration of S-phase was calculated according to the formula (Baserga and Nemeroff, 1962):

$$S = \frac{\begin{array}{c}(\text{number of BrdU labeled cells} \\ + \text{number of double labeled cells}) \\ \times (\text{duration between injections})\end{array}}{\text{number of tritium labeled cells}}.$$

*Duration of $G_2$ + M.* The interval $G_2$ + M was also determined using isolated fiber segment preparations from a total of 17 rats. A single intraperitoneal injection of BrdU was given and the interval between the injection and the time when the animal was killed was varied from 2 to 20 hr. For this assay, labeled satellite cell nuclei were examined on single, enzymatically isolated fibers (Kopriwa and Moss, 1971) to determine the time required after injection for the first nuclei to appear in late anaphase. A minimum of 300 BrdU-labeled nuclei were examined for each interval. Cell in late anaphase were identified as "doublets" of BrdU-positive nuclei (Fig. 1B) in contrast to the "singlets" seen 1–2 hr after injection (Fig. 1A).

*Duration of $G_1$.* The $G_1$ interval was determined by increasing the duration between the first ([$^3$H]thymidine) and second (BrdU) injections to a time when doubly labeled nuclei appeared. The shortest interval was longer than the length of S-phase to ensure that any doubly labeled cells were not a consequence of cells incorporating both labels during a single cell cycle. A single injection of [$^3$H]thymidine was given, after a duration of 16 ($n$ = 8), 17 ($n$ = 6) 18 ($n$ = 12), and 20 ($n$ = 6) hr. One hour after the last injection, the animals were killed. Fiber segments were prepared, immunostained for BrdU, and placed in autoradiography. The distribution of single and double labeled cells (Hume, 1989) was determined for each of the intervals. The length of G1 was estimated by subtracting the duration of $G_2$ + M from the time required to obtained doubly labeled cells.

### *Mitotic Behavior of Satellite Cells in Vivo*

*Continuous pump infusions: Electron immuno-microscopy.* In order to determine if all satellite cells had the same cycle time, their cell mitotic behavior was monitored in growing rats after 0.5, 2, 5, and 14 ($n$ = 3, animals each group) days of continuous infusion of BrdU. BrdU (10 mg/ml) was infused using an Alzet miniosmotic pump (2ML1). The pumps were filled and placed in 37°C saline for 4 hr to ensure that they were operating at the time of implantation. A small incision was made in the dorsal midline approximately 1 cm cranial to the base of the tail. The pumps were inserted through the incision into a subcutaneous position, leaving the proximal end of the pump resting at approximately the level of the scapulae. For longer infusion periods, the pumps were replaced on the seventh day.

At the conclusion of the pumping period the animals were killed by lethal injection of Beuthanasia D and perfused with sodium cacodylate-buffered 2% glutaraldehyde through the cannulated aorta. After fixation the EDL and soleus muscles were dissected free and embedded in Epon-Araldite without secondary fixation in osmium tetroxide. Thin sections were placed on nickel grids, etched with $H_2O_2$, and immunostained with mouse anti-BrdU (Becton Dickinson) followed by goat-anti-mouse (GAM) secondary antibody conjugated to 15-nm colloidal gold (Janssen Life Sciences Products). After secondary fixation in 2% glutaraldehyde, the sections were counterstained with uranyl acetate and lead citrate. The sections were methodically scanned at a magnification of 10,000× using grid bars for orientation. The number of colloidal gold-labeled and unlabeled satellite cell nuclei and myonuclei was tabulated and expressed as a percentage of each respective class of nuclei counted. A linear increase until saturation in the percentage of satellite cells that were colloidal gold positive would indicate that all cells were dividing in a muscle with approximately the same cell cycle time.

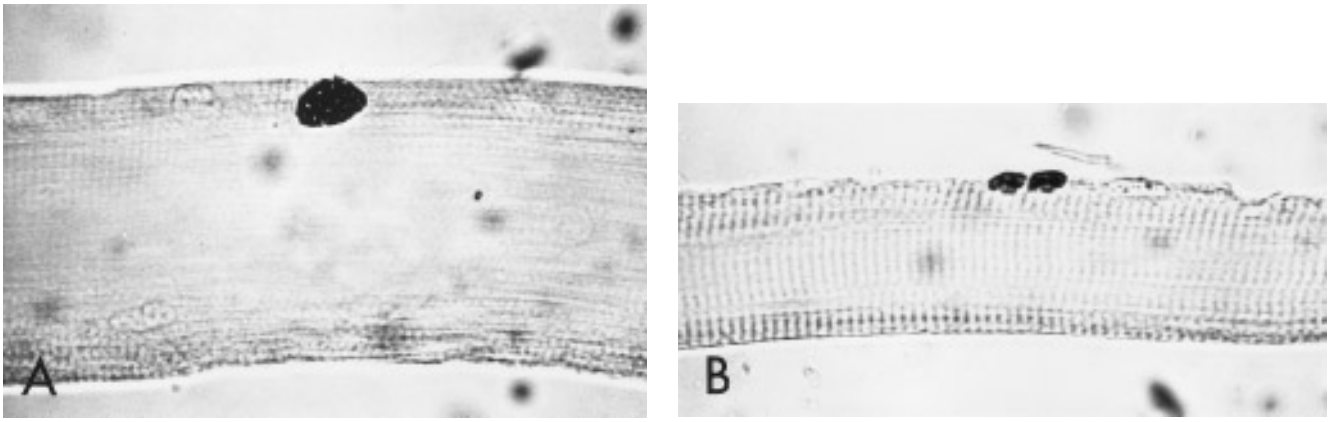


FIG. 1. Light micrograph of single fibers with a BrdU-positive nuclei as well as BrdU-negative nuclei. At 1–2 hr after injection, all labeled nuclei were single profiles (A). Beyond 2 hr after injection a fraction of the labeled nuclei appeared as doublets (B), indicating they had completed $G_2$ and were in a late stage of mitosis. Original magnification, ×500.

*Tandem pump infusions: Fiber segments of EDL and soleus.* Tandem pump infusions were used to determine (1) if new cells were continually entering the proliferative pool of satellite cells, and (2) the proportion of cells that divided more than once in a specific growth period. A group of four animals was used to monitor labeling following a continuous infusion period of 5 days with BrdU and then followed, after a 1-day hiatus of no labeling, with a continuous infusion period of 5 days using [$^3$H]thymidine. BrdU (10 mg/ml) or [$^3$H]thymidine (1 mCi/ml) was infused using an Alzet miniosmotic pump (2ML1) as described above. At the conclusion of infusion period, the soleus and EDL muscles were removed and processed for fiber segments as previously described. Fiber segments were examined for incidence of cells singly labeled with [$^3$H]thymidine, indicating that they entered mitosis for the first time during the last 5 days of the infusion period, and for incidence of cells that were doubly labeled, indicating that they divided more than once during the infusion period. Because the assay of labeled nuclei was carried out at the light microscopic level and labeled myonuclei were not distinguished from labeled satellite cells, the proportions of satellite cells, either cycling for the first time (single label) or cycling for more than one time (double label) during the experimental period, would be underestimated.

*Single injections of [$^3$H]thymidine.* In order to document that both soleus and EDL muscles were growing and adding myonuclei throughout the experimental period, a single injection of [$^3$H]-thymidine was administered to animals that were 30, 40, 50, 60, 70, 80, and 90 days old ($n$ = 3, each age). One hour after the intraperitoneal injection (2 $\mu$Ci/gram body weight) the animals were killed and the hindlimbs were removed and placed in Carnoy's fixative. After 24 hr the soleus and EDL muscles were prepared for autoradiography according to the procedure of Kopriwa and Moss (1971). The fiber segments were stained with Gill's hematoxylin. The numbers of total nuclei and labeled and unlabeled nuclei per unit length were tabulated.

*Statistical analysis.* The means obtained from the labeling experiments were compared using an unpaired, two-tailed $t$ test at a 95% confidence level.

## RESULTS

### *Soleus and EDL Muscles*

In each of the studies carried out, there was no difference in the pattern or percentage of labeled satellite cells in the soleus and EDL muscles. Data from EDL and soleus muscles were combined unless otherwise noted.

### *Cell Cycle Phases*

*S-phase.* The sensitivities of the two tracers was first determined by simultaneous intraperitoneal injections of [$^3$H]thymidine and BrdU. Under these conditions virtually all labeled nuclei were positive for both [$^3$H]thymidine and BrdU (data not shown). Less than 2% of nuclei had only a single label, presumably attributable to technical variation, showing that BrdU and [$^3$H]thymidine give similar labeling indices in skeletal muscles as in other tissues (Hume and Thompson, 1989; Qin and Willems, 1993). When the duration between injections was increased there was a corresponding increase in the number of singly labeled (BrdU or [$^3$H]thymidine) cells. A tabulation of the distribution of labeling from the muscles of a total of nine animals at two injection intervals indicated that the duration of the S-phase of satellite cells was 14.1 ± 1.4 hr.

*$G_2$ + M-phase.* The duration of $G_2$ + M was determined by single BrdU injections followed by survival periods of 2 to 20 hr. At 2 hr 100% of labeled nuclei were single profiles (Fig. 1A). However, at 4 hr nearly 4.7 % (Fig. 2) of labeled nuclei appeared as doublets (Fig. 1B), indicating that this duration was sufficient for cells labeled at the end of S-phase to have entered late anaphase. Thus, the interval of $G_2$ + M was determined to be approximately 4 hr. The percentage of labeled cells that were doublets remained at approximately the same level until 17 hr when the percentage had decreased to 1.1%. After 17 hr the percentage of labeled doublets remained below 1% (Fig. 2). Interestingly, beyond 17 hr doublets were no longer evident, suggesting that after mitotic division the daughter cells migrate away from each other, leaving no physical indication of the mitotic event.

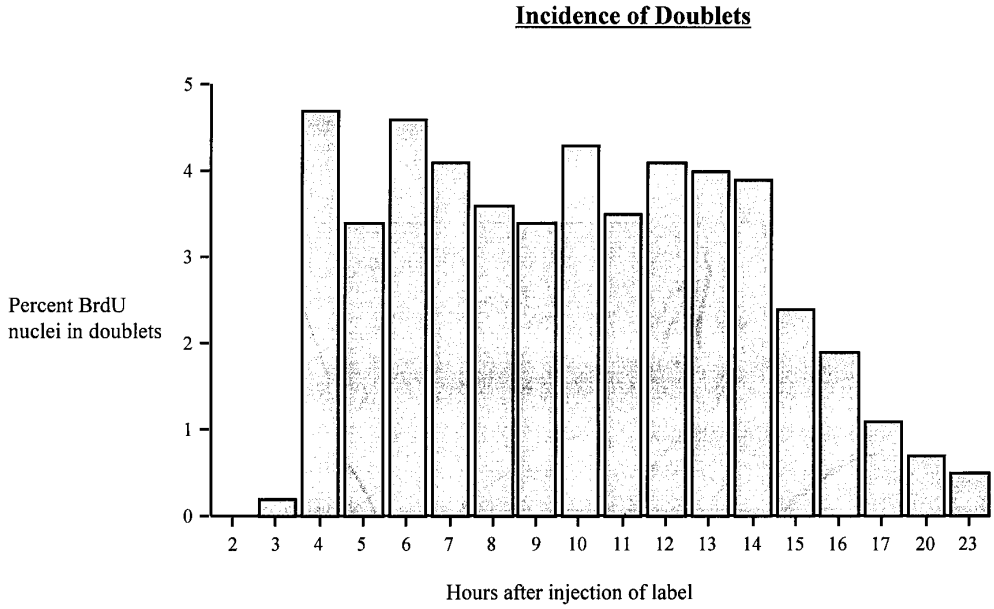


FIG. 2. The percent of labeled nuclei in late anaphase increases at 4 hr indicating the shortest time required for satellite cells to progress from the end of S-phase, through $G_2$, to the anaphase stage of mitosis. Each column represents the counts derived from a single animal.

### *Total Cycle and $G_1$-Phase Duration*

The duration of $G_1$ was determined by widely spaced injections of BrdU and [$^3$H]thymidine. After immunocytochemistry and autoradiography, fiber segments contained unlabeled nuclei, nuclei labeled with only [$^3$H]thymidine or BrdU, and nuclei labeled with both markers (Fig. 3). Doubly labeled satellite cells on fiber segment preparations exhibited a significant increase when the interval between injections was 18 hr (Fig. 4). This duration represents the shortest time needed for cells to progress from the end of the first S-phase when [$^3$H]thymidine was administered until the beginning of the second S-phase when BrdU was administered. Subtracting the duration of G2 + M (4 hr) indicates that the duration of G1 is approximately 14 hr. Although cell cycles were examined in satellite cells from both EDL and soleus muscles, no differences were apparent. The duration of the cell cycle of satellite cells in both glycolytic and oxidative muscles is the same and is approximately 32 hr.

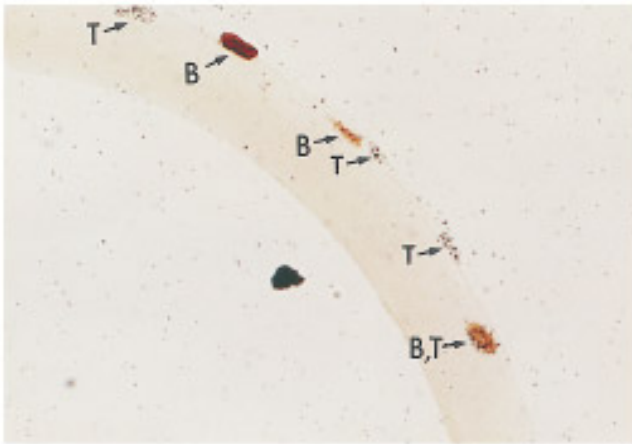


FIG. 3. Light micrograph of a portion of a fiber segment with BrdU- (B) and [$^3$H]thymidine- (T) positive nuclei. The labeled nuclei of each type are easily distinguished from each other as well as the doubly labeled nucleus (B,T). Original magnification, ×250.

### *Continuous Infusion LM and EM Studies*

*Labeling of the satellite cell and myonuclear population: Electron microscopy.* Continuous infusion of label was used to determine if all satellite cells in a muscle were mitotically active and dividing at the same frequency. Following colloidal gold immunostaining, nuclei that had incorporated BrdU during the infusion period were easily identified with the electron microscope. BrdU-positive nuclei were heavily labeled with gold particles (Figs. 5A and 5B), which tended to be located preferentially over the condensed chromatin of the nucleus. There was no difference in the percentage or pattern of labeling of satellite cells in the soleus or EDL at any time period studied. The combined data from EDL and soleus muscles after infusion periods of 0.5 to 14 days are summarized in Fig. 6 and showed no significant difference in the incidence of labeled satellite cells between 0.5 (28.5 ± 5.7%) and 2 (32.3 ± 3.1%) days of continuous infusion. In the interval from Day 2 to Day 5, labeling of the satellite cell popula-

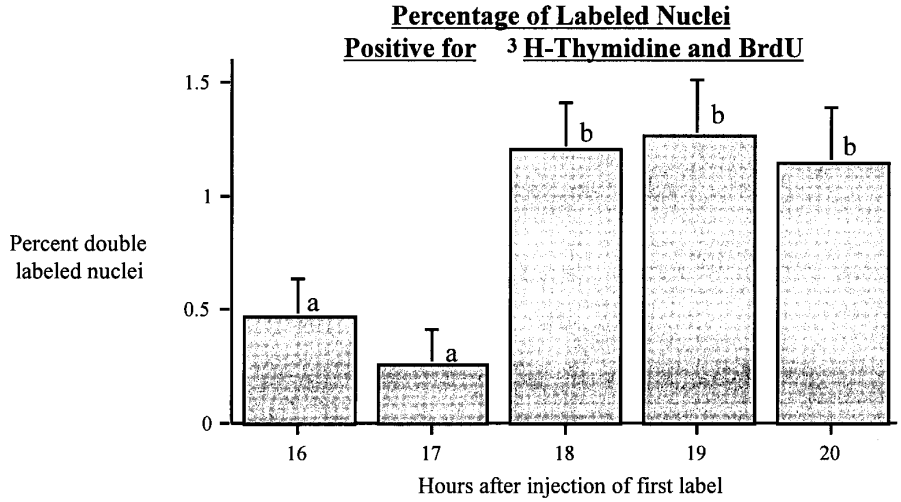


FIG. 4. Animals were first injected with [$^3$H]thymidine and then, after the indicated duration, with BrdU. There was a significant increase in the number of doubly labeled nuclei when the interval between injections was 18 hr (values with different superscripts are significantly different, $P < 0.05$). This was the shortest duration required for the initially labeled cells to traverse from the end of the S-phase when [$^3$H]thymidine labeled the cells to the beginning of the S-phase when BrdU labeled the cells ($G_2$ + M + $G_1$.) Labeling at 16 and 17 hr probably represents some reutilization of [$^3$H]thymidine and cells with different cycle (longer) times as the major cohort seen at 18, 19, or 20 hr.

tion increased significantly ($P < 0.0001$) to 82.2 ± 3.8%, reflecting a rate of increase of 16% per day. The percentage of labeled satellite cells did not change at 6 and 7 days of infusion, suggesting this subset of cells was fully saturated with label. The incidence of labeled satellite cells significantly increased ($P < 0.05$) between 5 and 14 days. At 14 days, 91.6 ± 2.5% of the satellite cells were labeled. The calculated rate of increase between 5 and 14 days was 1.08%/day.

Myonuclear labeling also exhibited a dramatic increase over the 14-day infusion period. At the end of the infusion period 18.6 ± 7.4 and 22.4 ± 9.3% of the myonuclei were labeled in the EDL and soleus muscles, respectively, showing that BrdU-labeled satellite cells were fusing with myofibers. At 14 days the percentage of myofiber nuclei that were satellite cells was 5.2 ± 0.63% in the EDL and 7.6 ± 1.6% in the soleus.

*Tandem continuous infusion of BrdU and [$^3$H]thymidine.* In order to obtain direct evidence that unlabeled satellite cells observed between 5 and 14 days in the continuous infusion experiments were entering the pool of dividing cells, BrdU was infused by osmotic pump for 5 days and then the pump was removed. After a 1-day hiatus a second pump containing [$^3$H]thymidine was implanted for 5 days. At the conclusion of the labeling period muscles were removed and fiber segments were prepared to examine the labeling characteristics of the myofiber nuclei. Under these labeling conditions 79.9 ± 2.64% of the labeled nuclei were positive for only BrdU, 13.9 ± 1.87% were positive for both markers and 5.9 ± 0.87% were positive for only [$^3$H]-thymidine. The last group, labeled with only [$^3$H]-thymidine, represented cells that divided for the first time during the second 5-day interval.

*Single injections of [$^3$H]thymidine.* The continuous infusion experiments suggested that after 5 days of continuous infusion there was a reduction in the uptake of label by satellite cells. Nuclear labeling after a single injection of [$^3$H]thymidine and the number of nuclei/mm were measured at 10-day intervals between 30 and 90 days of growth to determine when the soleus and EDL muscles stopped growing and if the reduced uptake of label could simply reflect a cessation of growth, rather than a difference in the mitotic behavior of some cells. The results of these experiments are presented in Table 1. The percentage of labeled nuclei in both the soleus and EDL muscles exhibited no decrease in labeling between 30 and 40 days of age and there was a corresponding increase in the total number of nuclei/mm, suggesting that nuclei were being added to the fibers during this portion of the growth period. Between 40 and 50 days there was no change in the percentage of labeled nuclei in the EDL ($P > 0.45$), although the soleus exhibited a significant ($P < 0.05$) reduction in labeling. The number of nuclei/mm increased steadily in both muscles, then stabilized in the EDL after 60 days and after 70 days in the soleus.

## DISCUSSION

### *Labeling with BrdU*

BrdU was used to label satellite cells during the S-phase of the cell cycle. Although BrdU inhibits terminal differenti-

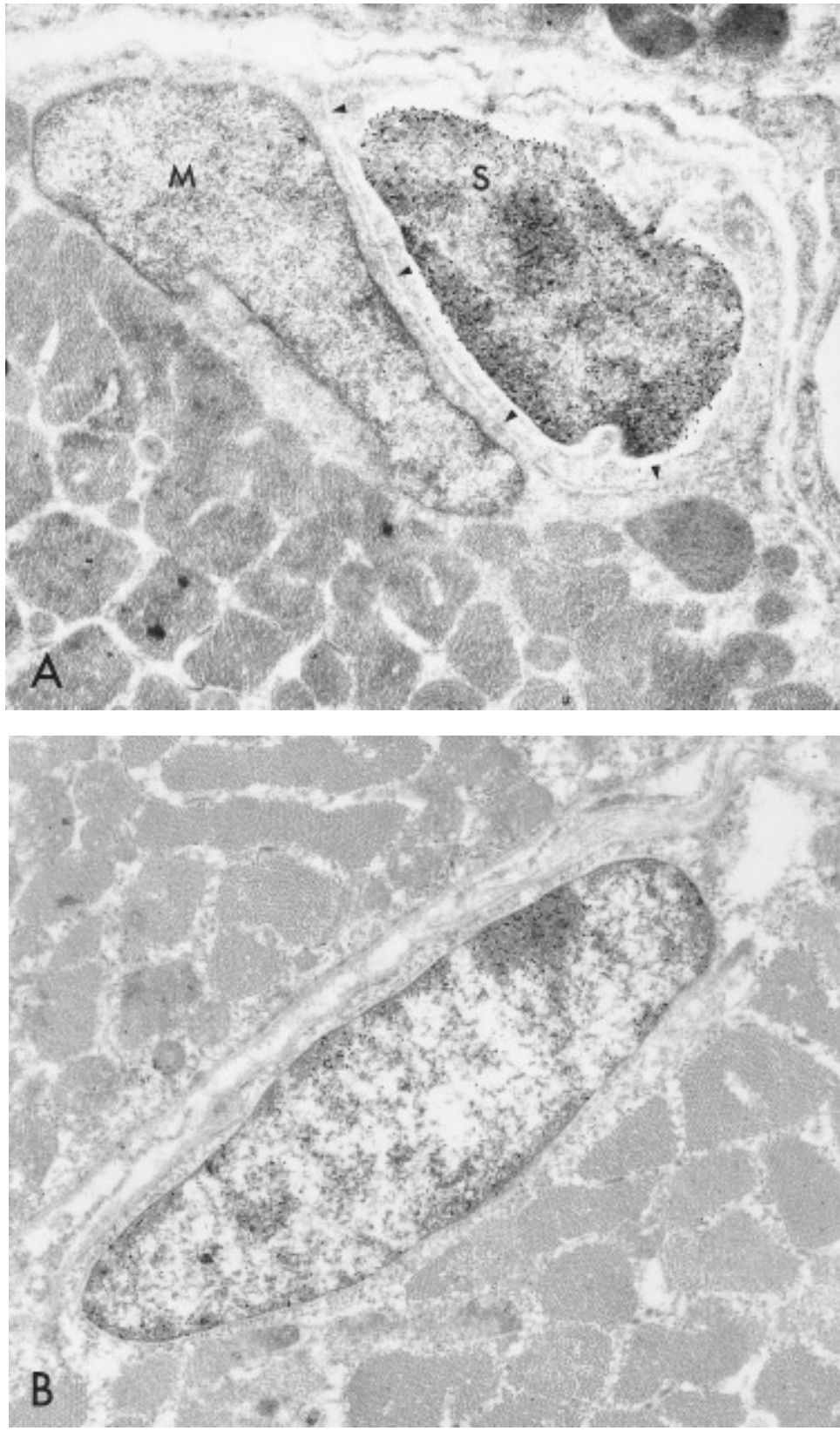


FIG. 5. Electron micrographs of BrdU-positive nuclei. In (A) a BrdU-positive satellite cell (S) is positioned adjacent to a BrdU-negative myonucleus (M). The interspace created by the membrane of the satellite cell and myofiber is marked with arrowheads. In (B) a myonucleus is well labeled with gold particles. The particles are preferentially located over condensed chromatin in both BrdU-positive nuclei. As was the case with all preparations that were used for quantitation, the background level of gold particles over the cytoplasm or negative nuclei in both A and B is very low. Original magnification, ×18,700.

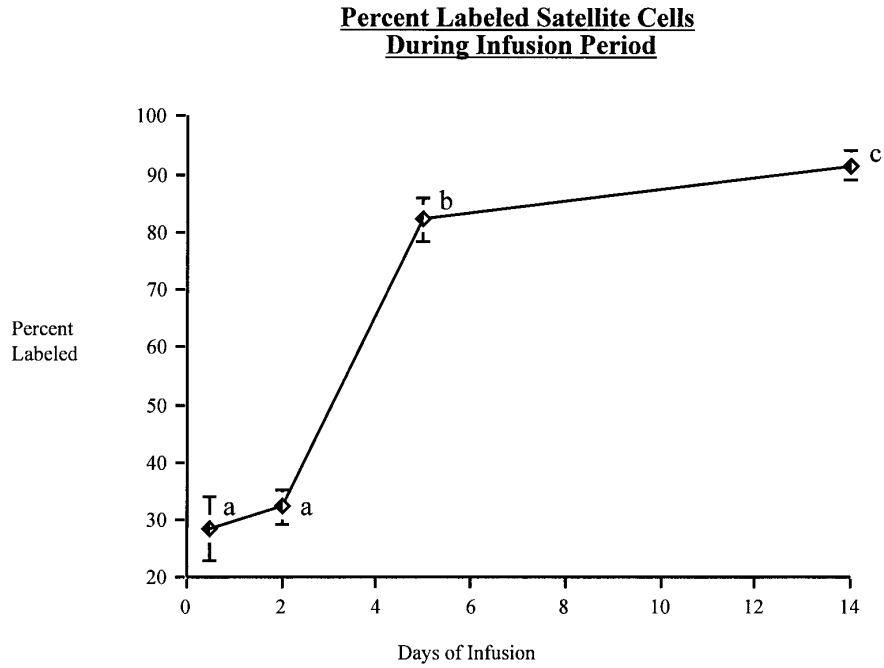


FIG. 6. The percentage of satellite cells positive for BrdU after infusion periods of varying durations. Each value with a different superscript is significantly different ($P < 0.05$). There was a decrease in the rate of increase of labeled satellite cells after 5 days of continuous infusion.

ation of myoblasts in culture (Bischoff and Holtzer, 1970), Labrecque *et al.* (1991) were able to obtain an immunocytochemical signal without altering myogenic cell behavior *in vitro* at concentrations below $0.5 \times 10^{-6}M$. The level of BrdU available to the cells in the present study could not be accurately calculated; however, the results suggest that *in vivo* concentrations of BrdU were at a level that permitted an immunocytochemical signal, yet did not alter satellite cell mitotic and fusion behavior. EM immunolabeling showed a 18–21% increase in labeled myonuclei, newly added as a consequence of satellite cell division and fusion. In addition, the percentage of satellite cells in muscles of treated animals was not different from those reported in previous studies of untreated animals (Gibson and Schultz, 1983). If BrdU blocked terminal differentiation at the dosages given, labeled myonuclei would have been abolished or greatly reduced and the percentage of satellite cells would have increased. These results suggest that BrdU is an appropriate marker to monitor mitotic activity of satellite cells *in vivo*.

### *Cell Cycle Duration*

Previous *in vitro* studies estimated the cycle time of satellite cells on isolated myofibers to be 12 hr (Bischoff, 1986, 1989, 1990); however, this duration was determined after

TABLE 1
Nuclear Changes and Satellite Cell Labeling in the Soleus and EDL Muscles during the Experimental Period

| | EDL | | Soleus | |
|---|---|---|---|---|
| Age in days | Nuclei/mm[a] | % Labeled nuclei[a] | Nuclei/mm[a] | % Labeled nuclei[a] |
| 30 | 51.4 ± 1.4 | 1.3 ± 0.07 | 104.5 ± 4.8 | 1.96 ± 0.98 |
| 40 | 57.1 ± 6.1 | 1.8 ± 0.25 | 117.3 ± 7.52 | 3.44 ± 0.54 |
| 50 | 54.1 ± 3.0 | 1.52 ± 0.60 | 124.4 ± 7.9 | 0.93 ± 0.64 |
| 60 | 62.3 ± 6.3 | 0.95 ± 0.25 | 143.5 ± 7.3 | 0.36 ± 0.25 |
| 70 | 68.3 ± 7.4 | 0.34 ± 0.10 | 175.1 ± 11.3 | 0.53 ± 0.08 |
| 80 | 71.3 ± 1.9 | 0.33 ± 0.12 | 175.7 ± 7.2 | 0.26 ± 0.15 |
| 90 | 63.9 ± 5.3 | 0.31 ± 0.08 | 176.3 ± 8.3 | 0.21 ± 0.07 |

[a] Mean ± SEM.

addition of exogenous growth factors or treatment of myofibers with myotoxic Marcaine had induced all cells to enter exponential growth. The method employed to measure the cell cycle time in the present study selected for those dividing satellite cells with the shortest cycle duration, making it unlikely that satellite cells in growing muscles *in vivo* have cell cycle durations less than 32 hr, but possible that some cells have cycle durations greater than 32 hr. The low level of doubly labeled cells prior to 18 hr in the G1-phase experiments (Fig. 4) could indicate the presence of cells with longer cycle times.

The duration of the cell cycle and, in particular, the length of S-phase (14 hr), shows why previous labeling studies, utilizing a single injection of tracer, obtained a relatively high labeling index. S-phase represents approximately 40% of the cycle duration. Since 80% of the cells are within this more rapidly dividing compartment, a labeling index as high as 30% of the satellite cell population could be expected after labeling muscles of immature growing animals. This figure is compatible with the reported values of 21% after a single injection in 1-day-old rats (Allbrook *et al.*, 1971) or 31% after two injections 4 hr apart in 20-day-old rats (Snow, 1978), or 20% after single injection of 30-day-old rats (Darr and Schultz, 1987).

The $G_2$ + M experiments illustrated a second feature of satellite cell behavior that helps to explain why labeled cells are seldom observed adjacent to one another. Satellite cells are reportedly evenly distributed along the length of a muscle fiber (Snow, 1981), with the possible exception of the region of the neuromuscular (Kelly, 1978, Wokke *et al.*, 1989). When the analysis of $G_2$ + M duration was carried out on fiber segments it was evident that satellite daughter cells quickly migrate away from one another after completion of a mitosis. By 17 hr after injection, the distribution of labeled satellite cells was never clustered or in pairs, suggesting possible mechanisms that help to disperse the cells on the myofiber surface, perhaps into domains according to information present on the myofiber surface.

### *Myonuclei-Producing Satellite Cells*

A cell cycle duration of 32 hr for producer cells appears consistent with the production of myonuclei reported in previous studies. Enesco and Puddy (1964) found nuclei increased from 779 to 2002 in myofibers between 36 and 90 days. The percentage of satellite cells at 36 days is approximately 12%, indicating that of the 779 total nuclei per fiber, 93 were actually satellite cells and would have been responsible for the observed increase in myonuclei over the period studied. The overall myonuclear increase for this growth period is on the order of 20 myofibers per day. If satellite cells had a cycle time of 12 hr and even if only 80% of the population were actively dividing and giving rise to myonuclei, the net production of myonuclei would be over 200 per day. If the cell cycle time were 32 hr as estimated in this study, the production of myonuclei by 80% of the population would approximate 43 per day at 36 days of age. Although this value is greater than the calculated 20 per day, the latter value is an estimate based on the entire 36- to 90-day growth period and assumes that growth occurs at the same pace throughout that period. In fact, myonuclear production may be high in muscles of young animals and reduced with maturity.

The most rapidly dividing cells in growing soleus and EDL muscles have a cell cycle time of 32 hr. Continuous infusion saturated this population in 5 days and further showed that approximately 80% of the satellite cells in a muscle are within this compartment. It follows that previous studies, such as the one carried out by Moss and Leblond (1971), labeled mostly producer cells and, given the high fusion index (50% at 48 hr) they observed, it is this subpopulation of satellite cells that is most intimately involved in the production of new myonuclei. Taken together these results suggest that the majority of mitotic divisions in growing muscle take place within the producer cell compartment and give rise to daughter cells that are destined to fuse with myofibers. The number of divisions that a producer cell undergoes before fusing with a fiber is unknown. The tandem continuous infusion experiments showed that approximately 15% of the cells labeled over 5 days with [$^3$H]thymidine were available for at least a second mitotic division during the subsequent 5 days of BrdU infusion. The relatively small number of doubly labeled cells suggests that only a small portion of the dividing satellite cells at any given time (of which the producer cells are the major component) are destined for more than a single mitotic division before fusing with a myofiber. Moss and Leblond (1971) reported that 48 hr following a single injection of [$^3$H]-thymidine, 50% of the labeled satellite cells had fused with their myofibers and that percentage increased to 65% after 72 hr. Taken together these results suggest that once satellite cells move from the reserve compartment to the producer compartment they will, on average, pass through a limited number of divisions before fusing with the fiber. This conclusion is consistent with the observation that satellite cells expressing myogenin appear to follow a program of limited proliferation (Yablonka-Reuveni and Rivera, 1994). Yablonka-Reuveni and Rivera (1994) suggested that myogenin expression in satellite cells marked a subset of cells that was nearing terminal differentiation. The notion of a subset of satellite cells with limited proliferations before fusion is also consistent with previous culture studies that showed (1) individual satellite cells are diverse with respect to the size of the colonies they generate and (2) the majority of satellite cells produce relatively small colonies (Schultz and Lipton, 1982). The producer cells identified in this study could possibly be the same subset of myogenin-positive satellite cells identified by Yablonka-Reuveni and Rivera (1994) and the small colony producers of *in vitro* studies (Schultz and Lipton, 1982). Finally, it is possible that some growth factors might modulate the total number of divisions before terminal differentiation (Mezzorgiorno *et al.*, 1993; Yablonka-Reuveni and Rivera, 1994) during regeneration or remodeling. It is not clear if some of the unfused daughter cells enter a quiescent state prior to fusion or another mitosis. Grounds and McGeachie (1987) have

shown that satellite cells fuse to form myofibers during a regeneration response after one or two divisions and proposed that committed, but unfused, satellite cells are present in adult muscle.

### *Reserve Satellite Cell Population*

Labeling kinetics during continuous infusion demonstrated the satellite cell population in growing muscles is composed of at least two compartments. Continuous infusion of BrdU labels all actively dividing cells of a population, but not resting or quiescent cells. This was clearly the case in the present study where all continually renewing cells of the gut were labeled after a 14-day infusion period (not shown). In contrast to the mucosal cells of the gut, two distinctly different labeling kinetics were evident in satellite cells. One population labeled to saturation within 5 days of continuous infusion. A second population, approximately 20% of satellite cells, accumulated label more slowly and was not completely saturated with label after 14 days of continuous infusion. There are at least three possibilities that might account for this result. One is that all satellite cells in this subset are dividing, but with a cell cycle time longer than 32 hr. A second is that some cells are true reserve cells and remain in $G_0$ during the growth period and are activated, for example, only during injury or hypertrophy in mature muscle. A third is that cells in this subset divide under appropriate signals but enter a quiescent $G_0$ state until growth of the fiber requires the addition of additional producer cells or myonuclei. The persistence of unlabeled cells after the very long infusion times used in this study makes it unlikely that all the cells in this compartment are simply dividing with a prolonged cell cycle time. The data are more consistent with cells in this compartment that spend varying periods of time in $G_0$. The length of time spent in $G_0$ would be dictated by the rate of growth and, therefore, the need for myonuclei. The labeling paradigm could not distinguish between the second and third possibilities or a combination of them.

One concern was that the difficulty in labeling satellite cells with continuous infusion was related to a change in the growth rate of the muscles. The presence of a population of cells in $G_0$ or with a greatly elongated cell cycle time might be expected in muscles approaching the end of their growth period and when the demand for additional myonuclei is reduced (Schultz *et al.*, 1978). The possibility that unlabeled satellite cells were present because of changes in growth pattern was investigated by the single injection experiments. There was no decrease in the percentage of cells labeled after a single injection of thymidine between 30 and 40 days of age. In fact, the number of nuclei per unit myofiber length continued to increase through 60 days of age in both EDL and soleus muscles. These results suggest the EDL and soleus muscles were growing over the entire duration of the infusion period and provide no evidence that unlabeled satellite cells observed during the infusion period were the result of muscle growth cessation.

The tandem double label pump experiments provided direct evidence that some but not necessarily all reserve satellite cells are mitotically active. In these experiments BrdU was continuously administered by mini-osmotic pumps for 5 days to label all dividing cells in the producer population. After a 1-day hiatus [$^3$H]thymidine was continuously administered for 5 days. This labeling paradigm produced [$^3$H]-thymidine-only (5%) labeled cells, which could only have been derived from the unlabeled reserve population. This feature of limited mitotic divisions in the reserve satellite cell population would conserve proliferative potential and allow for satellite cells in mature muscles that participated in postnatal myogenesis only to a limited extent. Similarly, this implies that some reserve cells have the ability to remain quiescent or continue cycling with a prolonged cycle duration and not differentiate despite mitogen-depleted culture conditions (Angello and Hauschka, 1994), suggesting a possible inherent program that functions to sustain the satellite cell population and its proliferative reserve. A program of this nature would be important in conserving the reserve population during regeneration and reestablishing the reserve population after repair is completed. There is already direct evidence that the preinjury distribution of cells within producer and reserve compartments is reestablished following regeneration (Schultz and Jaryszak, 1985).

### *Satellite Cells in the Soleus and EDL Muscles*

Oxidative fibers of the soleus accumulate more nuclei at a faster rate than EDL glycolytic myofibers during the growth period (Burleigh, 1977; Gibson and Schultz, 1983). Growth differences of oxidative and glycolytic fibers are probably not associated with any intrinsic properties of satellite cell population such as exists in the determination of fiber-type in avian muscles (Stockdale and Miller, 1987). There was no difference in any of the satellite cell labeling parameters examined in this study as a function of predominant fiber type in a muscle. Rather, cell-cycle analysis and labeling patterns after continuous infusion of BrdU demonstrated that all satellite cells exhibit the same characteristics despite the muscle source, suggesting that intrinsic and extrinsic controls regulating satellite cell mitotic behavior are the same in predominantly oxidative and glycolytic muscles. Specific fiber growth requirements for a larger or faster increase in the number of myonuclei are met by altering the number of satellite cells dividing rather than by adjusting the cell cycle. This hypothesis is consistent with the unequal distribution of satellite cells on myofibers of different types within a muscle (Gibson and Schultz, 1982; reviewed in Schultz and McCormick, 1994). Requirements over and above normal growth might be met by adjusting the total number of cells within a muscle that are cycling, and this parameter can be regulated by growth factors such as basic FGF (Yablonka-Reuveni and Rivera, 1994).

In summary, the satellite cell population on growing rat myofibers can be subdivided into two compartments. It is proposed that approximately 80% of satellite cells divide with a cell cycle time of 32 hr and are intimately involved in producing myonuclei. These producer cells divide an un-

known, but probably limited number of times before fusing with a myofiber. A compartment of cells, approximately 20% of the total, divides more slowly because of a longer cycle time and/or because many or most of the cells spend time in $G_0$. This compartment functions as a resource of cells with a large proliferative reserve. Mitotic divisions in the reserve population are presumably asymmetric, each giving rise to one daughter cell that maintains the reserve compartment, while the other daughter cell enters the producer cell compartment. The persistent unlabeled population after continuous infusion suggests that during normal growth the proliferative potential of the reserve population is conserved. In fact, the same mechanism may be present in regenerating muscle since the heterogeneity of colony-forming cells is maintained even after a regeneration response. That is, even in the highly stimulatory environment of a regeneration response, some cells must pass through only limited divisions (Grounds and McGeachie, 1987; E. Schultz, unpublished observations). Thus, both growth and regeneration are analogous to embryonic histogenesis, where some myoblasts, presumably presumptive (reserve) satellite cells, withdraw from cycling or cycle very slowly and do not fuse, while the remaining pool of myoblasts is mitotically active and fusion competent.

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