

Delayed somite formation in a quail line exhibiting myofiber hyperplasia is accompanied by delayed expression of myogenic regulatory factors and myosin heavy chain

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SUMMARY

A myofiber hyperplastic quail line P has been developed through selection for heavy body weight. Since the number of muscle fibers is determined early in development and skeletal muscle originates from somites, we compared somite formation and muscle-specific gene expression in P- and control C-line quail embryos. At 47 hours of incubation, C embryos had 18 somite pairs and P embryos had 14.3. By 72 and 120 hours, both lines appeared to be at the same stage of somite development. To determine whether the delay in the formation of the brachial somites was accompanied by alterations in muscle-specific gene expression, we conducted whole-mount *in situ* hybridization and immunofluorescence studies. At 47 hours of incubation, C embryos were expressing *qmf1* in the first 12 somites, while in P embryos only the first 7 somites showed *qmf1* activation.

Delays in expression were also observed for *qmf3* at 43 hours and for all three myogenic factors (*qmf1*, *qmf2* and *qmf3*) at 60 hours. At 65 hours, C embryos expressed myosin heavy chain in the first 15 somite pairs and P embryos in the first 7. At 72 hours, the transient delay in somite formation had disappeared and there was no lag in myosin heavy chain expression between the lines. The phase delay in brachial somite formation, myogenic factors and myosin heavy chain expression may be associated with the observed myofiber hyperplasia in P-line quail by allowing an increase in the muscle stem cell population.

Key words: somite, *qmf1*, bHLH, myogenic factors, myogenesis, myosin heavy chain, *Couturnix*, quail, hyperplasia

INTRODUCTION

Despite recent advances in understanding the molecular and cellular mechanisms underlying muscle cell determination and differentiation (Blau et al., 1985; Emerson, 1990; Olson, 1990; Weintraub et al., 1991; Wright, 1992), many questions regarding what controls the number of cells that form muscle in an organism remain unanswered. Muscle mass in adults is determined by both myofiber number and size. Increases in muscle can occur by both an increase in myofiber diameter (hypertrophy) and number of myofibers (hyperplasia). The ability to increase muscle mass by augmenting the work load of a muscle has proven to be an important model to study muscle and myofiber hypertrophy (Goldberg et al., 1975). Models to study myofiber hyperplasia exist in cattle and swine (Swatland and Kieffer, 1974; King and Menissier, 1982; Swatland, 1982, 1984; Quinn et al., 1990), but the difficulty in obtaining embryos and the limited embryological information available on these species make them experimentally difficult to work with.

However, a myofiber hyperplastic quail line P has been

developed through quantitative trait selection for heavier body weight (Marks, 1978a,b, 1988). The P line has undergone 88 generations of selection for heavy body weight and at 4 weeks of age is three times heavier than the control C line. An increase in body weight is first detected at 6 to 8 days of embryonic development (Lilja and Marks, 1991). At 10 days post-hatch, P-line quail had a 3.5-fold heavier pectoralis muscle, a 3-fold increase in DNA content with no change in the protein/DNA ratio and a 1.6-fold increase in the number of myofibers in the semimembranosus muscle (Fowler et al., 1980).

Myofiber formation takes place during embryonic development and no significant increase in the number of myofibers occurs after hatching or birth in birds and mammals (Swatland and Kieffer, 1974; Swatland, 1982, 1984; Konigsberg, 1986). Myofibers form through fusion of myoblasts that are derived from precursor cells in the somites (Christ et al., 1974, 1977; Chevalier et al., 1977; Keynes and Stern, 1988; Stern et al., 1988). In avian embryos, somites originate from the anterior end of the segmental plate and somitogenesis progresses in a cranial

to caudal direction. The somite is initially composed of columnar epithelial-like cells arranged radially around a small lumen. The ventromedial part of the somite gives rise to the sclerotome. The dorsolateral cells form the dermomyotome that further matures into the dermatome and myotome (Kaehn et al., 1988). As shown by microsurgery and quail-chicken chimeras (Ordahl and le Douarin, 1992), cells from the medial halves of somites give rise to muscles in the back, while cells from the lateral halves migrate away to form muscles in the limbs.

The *myoD* family of genes encoding *myoD*, *myf5*, *myogenin* and *mrf4/herculin* (Davis et al., 1987; Braun et al., 1989, 1990; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright et al., 1989; Clark et al., 1990; Miner and Wold, 1990) has recently been isolated and shown to play a role in muscle cell determination and differentiation (Emerson, 1990; Olson, 1990; Weintraub et al., 1991; Wright, 1992). These genes encode basic helix-loop-helix transcription factors capable of activating muscle-specific genes. Forced expression of these factors can also alter cell fate and activate the expression of muscle-specific genes in non-muscle cells (Weintraub et al., 1989; Miner and Wold, 1990). Homologs to three of the four genes have been cloned in quail (de la Brousse and Emerson, 1990; Pownall and Emerson, 1992). Their expression in somites is ordered and occurs in a cranial to caudal direction. *Qmf1* is the quail homolog of *myoD* and is first detected in the medial half of somites prior to myotome formation. Its expression is followed by *qmf3* (*myf-5*), *qmf2* (*myogenin*) and other muscle-specific genes such as *troponin I*.

In this report, we describe the results of experiments using a growth-selected quail line to investigate the mechanisms involved in myofiber hyperplasia. Because the number of myofibers is determined early in development and skeletal muscle cells originate from somites, we analyzed somite formation and activation of muscle-specific genes in embryos from the growth-selected P line that displays myofiber hyperplasia. We found that the fast growing P line undergoes a delay in early somite formation and muscle-specific gene expression.

MATERIALS AND METHODS

Embryo collection and staging

Eggs from Japanese quail (*Coturnix coturnix japonica*) lines P and C were obtained from the Southern Regional Poultry Genetics Laboratories at the University of Georgia and stored at 4–11°C for less than 1 week. Incubation was conducted at 37°C in a humidified chamber. Embryos were collected at different times, dissected away from the area pelucida, staged according to modified Hamburger and Hamilton (Hamburger and Hamilton, 1951), fixed for 24 hours with 4% paraformaldehyde in PBS and stored at –20°C in 70% ethanol until use.

In situ detection of mRNA

The *qmf1* (cC509), *qmf2* (cC527) and *qmf3* (cC528) cDNAs were generously provided by Dr Charles Emerson. Probe synthesis was based on the protocols from the Genius™ system (Boehringer Mannheim Biochemicals) and whole-mount detection of *qmf1* has been described in detail (Coutinho et al., 1992). Briefly, anti-sense or sense RNAs were synthesized using T7 or T3 RNA polymerase,

respectively, in the presence of digoxigenin-labeled UTP. Fixed whole embryos were prehybridized in hybridization solution (50% formamide, 4× SSC, 1× Denhardt's solution, 0.5 mg/ml heat-denatured herring sperm DNA, 0.25 mg/ml yeast tRNA and 10% dextran sulfate) for 2 hours at 63°C. Hybridization was performed in fresh hybridization solution plus 0.25 µg/ml of digoxigenin-labeled RNA probe for 48 hours at 63°C. Embryos were then rinsed and incubated in fresh hybridization solution (no probe) for 3 hours at room temperature and then for 12 hours at 63°C. Embryos were finally washed successively for 20 minutes each at room temperature in 80% hybridization solution (Hyb)/20% PBT, 60% Hyb/40% PBT, 40% Hyb/60% PBT, 20% Hyb/80% PBT and three washes for 1 hour each in PBT. Immunological detection was performed using an anti-digoxigenin-alkaline phosphatase conjugate and a color reaction (NBT and X-phosphate) as described in the Genius™ system protocol from Boehringer Mannheim (Indianapolis, IN).

Immunofluorescence detection of myosin heavy chain proteins (MHC)

Whole-mount detection of MHC has been described in detail (Coutinho et al., 1992). Briefly, non-specific protein interactions were blocked by incubation in 5% calf serum in PBS plus 0.3% Triton X-100 (PBTCS) at 37°C for 30 minutes. MHC was detected by incubation at 37°C for 30 minutes with MF-20, an IgG_{2b}K monoclonal antibody to myosin heavy chain (Bader et al., 1982). MF-20 hybridoma supernatant was concentrated 20-fold by precipitation with 50% ammonium sulfate and used at a 1:20 dilution in PBTCS. Embryos were rinsed three times in PBTCS and incubated at 37°C for 30 minutes in PBTCS plus goat anti-mouse IgG conjugated to the fluorescent dye Cy5™ (Jackson ImmunoResearch Laboratory, West Grove, PA). Embryos were finally washed several times in PBT and MHC staining visualized on a Bio-Rad MRC-600 confocal laser scanning microscope at an excitation wavelength of 647 nm. In some experiments, MHC antibody was detected by goat anti-mouse conjugated to alkaline phosphatase and the blue color reaction.

Yolk surface temperature profiles during reinitiation of development

An approximate 3×5 mm rectangle was cut into quail eggshells at one-third the distance from the blunt end. The eggshell was pried open, a thermistor probe (XSI P/N080) inserted onto the surface of the yolk, the eggshell closed, expressed albumen wiped off, and the eggshell sealed with hot glue. Eggs containing thermistors remained at 4°C for 1 hour before placement into an incubator operating at 37.5°C. Two P- and two C-line eggs per replicate (*n*=8) were placed in the center of the incubator in a single row perpendicular to the air circulation pattern. Temperature was recorded with a RyanTemp Mentor every second for 60 minutes by which time all eggs had reached incubator temperature. Temperature-time data were retrieved into a graphic program (Sigma-Plot) and plotted. The area under the temperature curve was quantified using a digitizing tablet and Sigma-Scan software.

Cell cycle measurements

The procedure used was modified from the method described by Kenneth Bauer in the Annual Course of Flow Cytometry Manual. Nuclei were obtained from individual unfixed embryos by vortexing in a low-salt staining solution (3% w/v PEG 8000, 5 µg/ml Hoescht 33238, 10 µg/ml ribonuclease A, 0.1% Triton X-100, 4 mM citrate buffer, pH 7.2) followed by 20 minutes of incubation at 37°C. Nuclei were then diluted with 1 volume of salt solution (3% w/v PEG 8000, 5 µg/ml Hoescht 33238, 0.1% Triton X-100, 0.4 M NaCl, pH 7.2) and stored at 4°C overnight. Hoescht fluorescence dye was excited using an argon laser at 125 mW of UV

power. Fluorescence was collected through a 320 nm filter on a EPICS 753 flow cytometer (Coulter Electronics Hialeah, FL). Data were analyzed using Autoanalysis option of the Modfit software (Verity Software House, Topsham, ME).

RESULTS AND DISCUSSION

Somite number in P- and C-line embryos

As can be seen in Fig. 1 ($n > 10$ /time point), somites began to form after 30 hours of incubation in both lines. During the next 10 hours, C and P embryos appeared to develop at the same rate, as measured by somite pairs. At 44 hours, however, C embryos had 12.9 ± 1 and P embryos 11.2 ± 1 somite pairs, and by 47 hours, C had 18.0 ± 2 somite pairs and P had 14.3 ± 2 . These differences were significant at $P < 0.01$. This ~4 somite pair delay, nevertheless, was transient. The P embryos lagged 2.4 somites behind the C embryos at 59 hours ($P < 0.01$) and by 72 and 120 hours (not shown on figure), both lines were at the same stage of development. These results confirm and extend an earlier observation that embryos from the P line at the 66th generation of selection had a reduced number of somites at 42 hours of incubation (Lilja and Olsson, 1987).

The increase in body weight and muscling observed in P-line quail is also accompanied by a 40% increase in egg weight. To determine if the increase in egg size could slow the initial development of P embryos, we monitored the yolk surface temperature during reinitiation of development. When started at 4°C, C-line eggs reached 37°C in 60 minutes and P-line eggs lagged no more than 2 minutes behind. This difference is not likely to affect embryos at the time points used in this study and is within typical sampling time error. This conclusion is also supported by the observation that at 30 and 40 hours of incubation, P- and C-line embryos were at the same stages in somite development (Fig. 1).

Expression of myogenic regulatory factors and MHC in P and C embryos

The somites that undergo delayed formation are the brachial ones and these contribute to muscle formation in the limbs and the thorax region (Chevallier, 1979). To determine whether the delay in somite formation in the P line was accompanied by a delay in gene expression, we measured the expression of *qmfl*, which is the earliest known marker of skeletal muscle development (de la Brousse and Emerson, 1990; Pownall and Emerson, 1992). Detection of *qmfl* expression is shown in Fig. 2 in a whole-mount quail embryo at 47 hours of incubation. *Qmfl* signal appears as a thin blue line along the medial side of somites (Fig. 2, panel A). *Qmfl* activation progresses in a cranial-to-caudal orientation and the most caudal somites, recently formed, have not activated *qmfl* (Fig. 2, panel B). We employed a whole-mount in situ procedure for quail embryos (Coutinho et al., 1992), which permitted an easy comparison between the lines by counting the number of *qmfl*-positive somites. It does not require tedious serial dissection of the embryo and avoids comparison of sections obtained at different planes. As shown in Table 1A, studies conducted with embryos incubated for 47 hours revealed that *qmfl* was

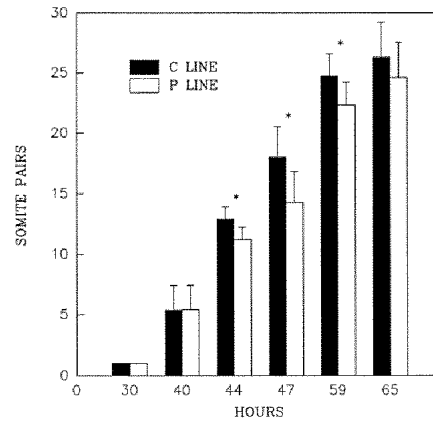


Fig. 1. Somite pairs present in C- and P-line embryos during early development. At least 10 embryos per line were used for each time point. Error bars indicate standard error of treatment mean and * indicates statistical significance at $P < 0.01$ level using the F test.

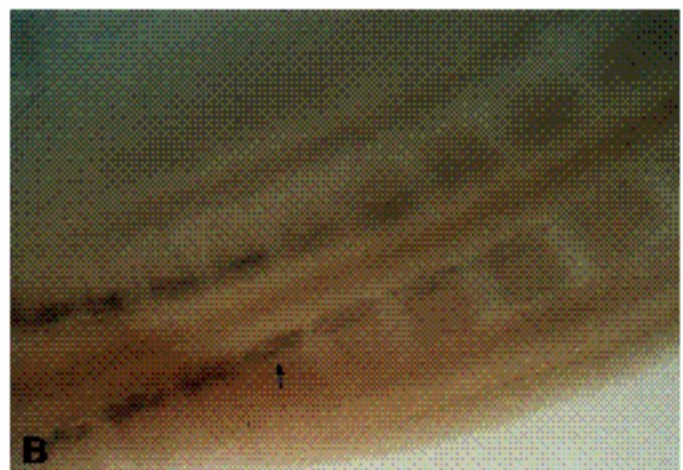
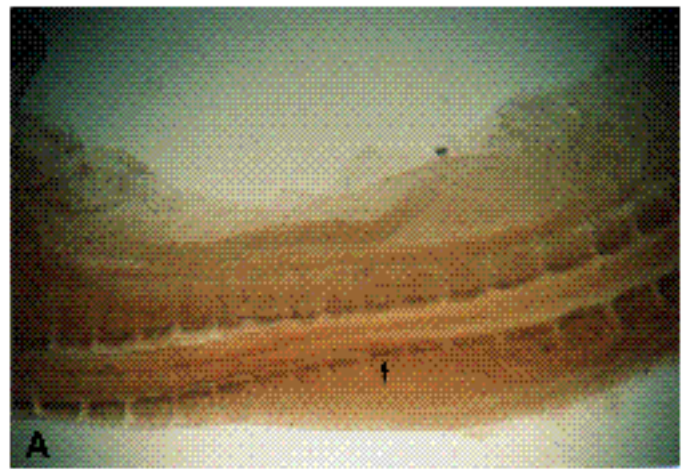


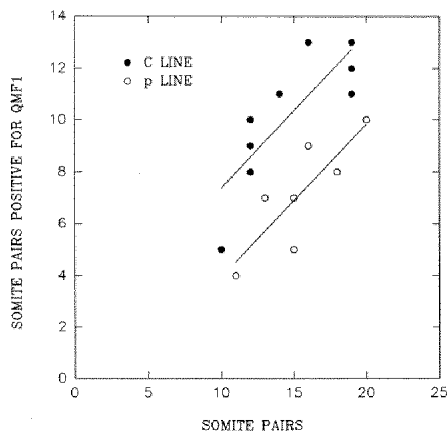
Fig. 2. Whole-mount in situ detection of *qmfl* transcripts after 47 hours of incubation from a P-line embryo containing 22 somite pairs at 63 \times magnification (A). Somites shown in the panel are 6-21. (B) The same embryo is shown at 160 \times of magnification. Embryos are oriented in a cranial to caudal direction from left to right. The last somite seen in B is somite 22. Arrows locate *qmfl* signal in somite 15 in A and somite 18 in B.

Table 1. Expression of *qmf1* (A) and *qmf3* (B) in C and P line embryos at 47 and 43 hours of incubation, respectively*

Quail line	n	Number of somites	Number of positive somites	% Positive
(A) <i>qmf1</i> expression				
C	3	19.0±1	12.0±1.4	63
P	4	14.8±1	7.0±1.4†	47
(B) <i>qmf3</i> expression				
C	6	15.3±0.7	9.2±1.2	60
P	6	13.7±0.7	6.8±1.2‡	50

*Results expressed as treatment mean ± standard error of the treatment mean.
†‡Denote statistical significance at $P<0.01$ (†) and $P<0.06$ (‡) using the F test.

expressed in the first 12 of 19 somites (63%) in C embryos and in the first 7 of 15 somites (47%) in P embryos. These results indicate that at 47 hours P embryos were delayed not only in somite number but also in *qmf1* expression, and perhaps in the muscle differentiation program. To address whether the delay in *qmf1* expression could be explained by the delay in somite formation, we measured *qmf1* in embryos having 10 to 20 somite pairs. The number of *qmf1*-positive somites was plotted against the total number of somites in the embryo. As shown in Fig. 3, when C and P embryos with the same number of somites are compared, a delay in *qmf1* activation was still observed. Therefore, the lag in *qmf1* activation in P embryos is not solely a result of the lag in somite formation. Although the color reaction employed for signal detection does not allow precise quantification of signal, we also observed less staining in those somites that had activated *qmf1* in P embryos. The analysis of *qmf3* expression in embryos at 43 hours (14-15 somites) of incubation is shown in Table 1B. This myogenic factor, like *qmf1*, was first detected in the medial half of somites (data not shown). For C embryos, *qmf3* transcripts were detectable in 60% of the formed somites while only 50% of the P embryo somites were positive. To include more of the brachial somites in our analysis, we studied the expression of the three known quail myogenic factors in

**Fig. 3.** Somite pairs positive for *qmf1* transcripts plotted against the total number of somite pairs formed. Linear regression lines were obtained with the SigmaPlot program.**Table 2. Expression of *qmf1* (A), *qmf3* (B) and *qmf2* (C) in C and P line embryos at 60 hours of incubation***

Quail line	n	Number of somites	Number of positive somites	% Positive
(A) <i>qmf1</i> expression				
C	4	24.8±1.8	22.8±2.3	92
P	4	21.5±1.8	19.0±2.3†	88
(B) <i>qmf3</i> expression				
C	3	25.7±1.5	18.0±2.5	70
P	3	22.7±1.5	13.3±2.5†	59
(C) <i>qmf2</i> expression				
C	8	24.5±2.0	13.9±2.3	57
P	8	21.6±2.0	10.3±2.3‡	48

*Results expressed as treatment mean ± standard error of the treatment mean.
†‡Statistical significance at $P<0.08$ (†) and $P<0.01$ (‡) using the F test.

embryos at stages 14 and 15 (22-25 somites). The results for *qmf1*, *qmf3* and *qmf2* expression in embryos at 60 hours of incubation are shown in Table 2. Our results confirm the observations of Pownall and Emerson (1992) that the expression of the quail myogenic factor genes occurs sequentially and in a cranial-to-caudal fashion. In C embryos, we detect *qmf1* transcripts in 92% of the somites formed. *Qmf3* was detectable in 70% while *qmf2* was expressed in only the more mature somites (57%). It can also be seen that the expression of all three myogenic regulatory factors is delayed in P embryo somites.

We next investigated the expression of MHC, a later marker of muscle differentiation in somites (Bader et al., 1982). MHC protein was detected by immunochemical staining of whole-mount embryos and confocal laser scanning microscopy (Coutinho et al., 1992). One advantage of this procedure is that the sample can be optically dissected improving localization of signal. In Fig. 4, MHC staining is shown on C (panel A) and P (panel B) embryos after 65 hours of incubation. The C embryo shown had 28 somites formed and the first 18 were positive for MHC (64%), while the P embryo had 29 somites formed and the first 14 were positive for MHC (48%). In Table 3, we compare the number of somites positive for MHC in P and C embryos after 65 and 72 hours of incubation. At 65 hours (20-30 somites), there was a delay in MHC expression in P embryos. In C embryos, 15 of 26 somites (57%) were positive for MHC compared to 7 of 23 somites (31%) in P embryos. Even when embryos with nearly the same number

Table 3. Expression of MHC in C and P line embryos at 65 (A) and 72 hours (B) of incubation*

Quail line	n	Number of somites	Number of positive somites	% Positive
(A) 65 hours				
C	13	25.9±3.6	14.7±6	57
P	10	22.9±3.6	7.0±6†	31
(B) 72 hours				
C	4	34.0±1.3	21.5±2.9	63
P	4	33.8±1.3	21.3±2.9†	63

*Results expressed as treatment mean ± standard error of the treatment mean.

†Denotes statistical significance at $P<0.05$ using the F test.

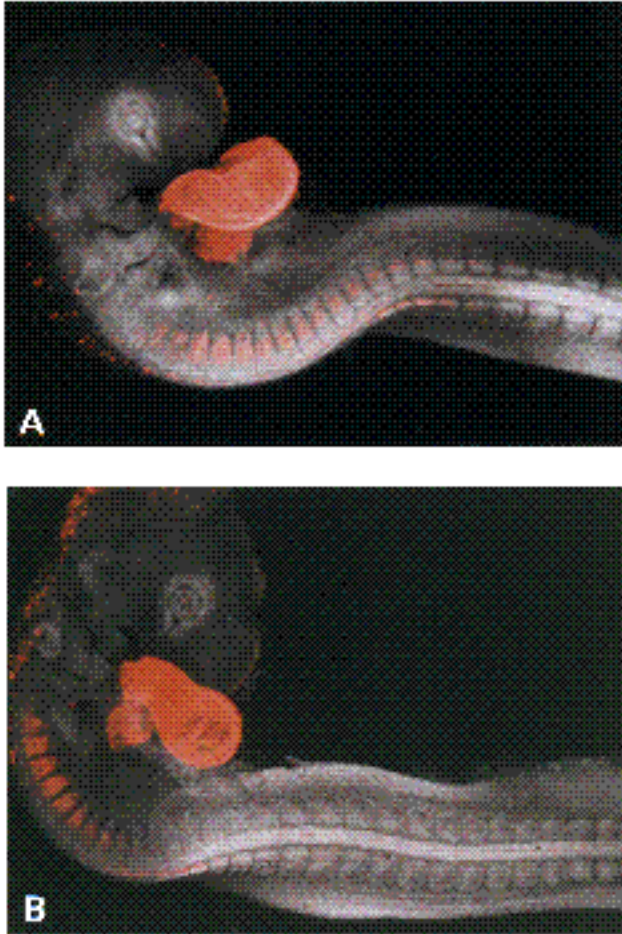


Fig. 4. Whole-mount detection of MHC proteins (shown in red) by immunocytochemistry in embryos incubated for 65 hours from the control line (A) and the P line (B). The control embryo contains 28 somites and the first 18 are positive for MHC. The P-line embryo contains 29 somites and the first 14 are positive for MHC. MHC staining was detected on a confocal laser scanning microscope and the optical sections were selected to show MHC expression in the positive somites. MHC signal was collected at an excitation wavelength of 647 nm and overlaid on fluorescent images of neutral-red-stained embryos collected at 568 nm.

of somites were compared, the P-line embryos were still delayed in MHC expression, as can be seen in Fig. 4. However, by 72 hours of incubation, when both lines had the same number of somites formed (34), there was no detectable difference in MHC expression. These results underscore the transient delay in somite formation observed in P-line embryos. The window for delay of both somite formation and myofactor gene expression was between 44 and 65 hours of incubation, the time at which brachial somites were developing. As embryogenesis proceeds, P-line embryos enter an accelerated growth phase (Lilja and Olsson, 1987) and by 144 hours they have an increased body weight.

Delay in somite formation and myofiber hyperplasia in the P line

It is possible that delayed somite formation and activation of myogenic factors in the P-line embryos may be involved with an increase in the muscle precursor cell population. Once a cell migrates from the dermomyotome to the myotome, it exits the cell cycle and initiates the myogenic program (Langman and Nelson, 1968; Kaehn et al., 1988).

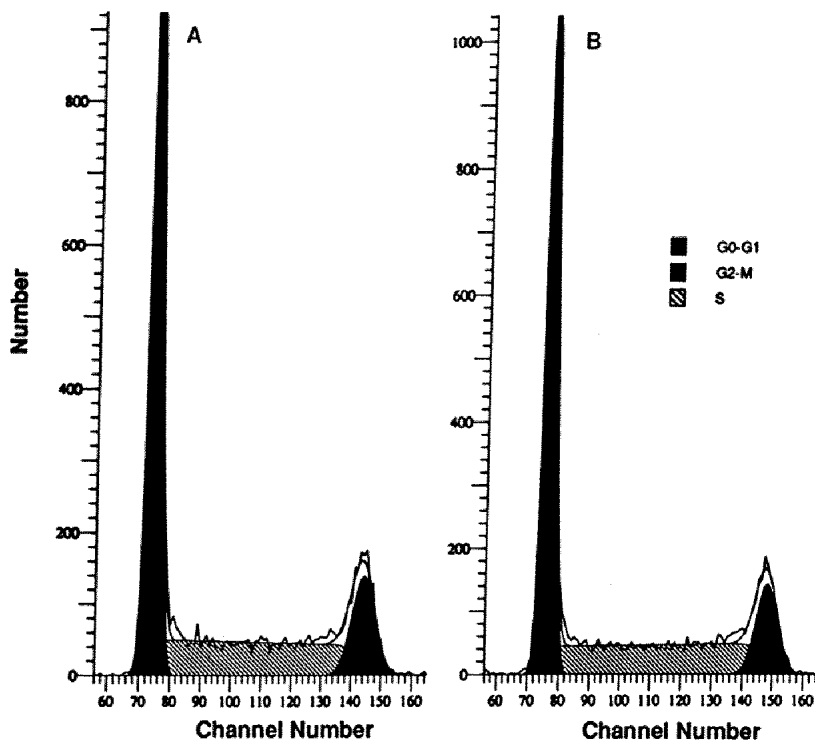


Fig. 5. Representative cell cycle phase distribution of whole embryos from C (A) and P lines (B). Embryos were collected at 47 hours of incubation, individually dispersed and nuclei analyzed by flow cytometry after staining with Hoescht 33238.

Table 4. Cell cycle phase distribution in C and P line embryos at 47 hours of incubation*

Quail line	n	% G0-G1	% G2-M	% S
C	11	50.8	14.7	34.5
P	9	50.9	14.7	34.4

*Data from each embryo were averaged and expressed as a percentage of the cells in each phase of the cell cycle.

Perhaps a delay in cell differentiation can allow additional rounds of cell division in the dermomyotome. As one test of this hypothesis, we determined the cell cycle phase distribution by flow cytometry of nuclei from whole embryos incubated for 47 hours. Typical profiles are shown in Fig. 5 for C (Panel A) and P (Panel B) lines. The average percentages of cells in each phase of the cycle for at least 9 embryos from each line are shown in Table 4. It can be seen that there was no detectable difference in the cell cycle distribution between the lines, indicating that there was no overall delay operating throughout the P embryo. Possibly, only the cells involved in somite formation have an altered proliferation rate, and since these are a small percentage of the whole embryo, we could not detect any difference between lines.

In this report, we have shown that a quail line P displaying muscle hyperplasia serves as a useful model system to study the control of muscle formation. The hyperplastic line P undergoes a delay in the formation of the brachial somites. There is also a delay in the somitic activation of the myogenic factors and MHC expression in this line reflecting a delay in the onset of myotome differentiation. We suggest that this delay may allow muscle precursor cells to proliferate for a longer time, resulting in an increase in the pool of myogenic cells.

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