

Expression of Phospholamban mRNA During Early Avian Muscle Morphogenesis Is Distinct From That of α -actin

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ABSTRACT We have studied the expression of phospholamban during the early development of chick embryos by *in situ* hybridization and have compared it to that of α -cardiac and α -skeletal actin. In adult cross-striated muscles there is only one phospholamban gene and it is expressed exclusively in the heart and slow muscles. In the heart phospholamban transcripts were first detected at stage 14 in the region of presumptive ventricle and at stage 20 in the atrium. In the myotomal portion of the somites phospholamban mRNA was first detected at stage 20, which lagged behind the appearance of the α -actins. In the limb rudiments all three mRNAs were barely detectable through stage 24, but increased by stage 28+. However, quantitative analysis of signal intensity at stage 28+ indicated that less phospholamban mRNA is present in the limb bud than in the myotome since for phospholamban the ratio of the signal density in the myotome to that in the limb rudiments was about twice the value of the ratio determined for the α -actins. Northern blot analysis of embryonic day 11 chick fast pectoralis muscle showed that phospholamban mRNA was not detected *in vivo* while α -cardiac actin mRNA was. Moreover, no phospholamban mRNA was detected in primary cultures derived from pectoralis muscle of the same age. In concert with previous observations that phospholamban is not detectable at stage 30–32 in wing or thigh muscle, these results suggest that phospholamban mRNA is expressed independently of the α -actins in the limb buds during early myogenesis.

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Key words: Phospholamban, α -Actin, Chick embryo, Muscle morphogenesis, Hybridization *in situ*

INTRODUCTION

Phospholamban is a pentamer of identical subunits that regulates the Ca^{2+} -ATPase of the sarcoplasmic reticulum (Tada and Katz, 1982). In adult animals it is expressed in cardiac and slow, but not fast, skeletal muscle cells, and some smooth muscle cells (Raeymaekers and Jones, 1986). Under β -adrenergic stimulation, phospholamban becomes phosphorylated by a cAMP-dependent protein kinase, and this results in increased Ca^{2+} -ATPase activity and enhanced efficiency

of muscle relaxation (Edes and Kranias, 1989). Ca^{2+} -ATPase protein has been observed by antibody staining to appear in the developing chick heart at stage 10 (Jorgensen and Bashir, 1984), coincident with the origination of heart contractility. Phospholamban protein was detected by induction of phosphorylation in day 4 chick hearts (\pm stage 23) (Will et al., 1983). These authors presented evidence that genetic regulation of phospholamban and Ca^{2+} -ATPase is not identical.

Cardiac and skeletal myocyte precursor cells are derived from two separate lineages. The cardiogenic lineage forms at stage 4 by commitment of cells of the lateral plate mesoderm (Gonzalez-Sanchez and Bader, 1990), whereas the skeletal muscle precursors are derived from somitic mesoderm and begin to differentiate at about stage 13 (Holtzer et al., 1957). In the initial stages of myogenesis, cardiac isoforms of numerous proteins are expressed not only in the heart, but also in primordia of future skeletal muscles of both fast and slow fiber types. Thus far this has been found to be the case for myosin heavy chain, light chains 1 and 2, α -actin, troponin-T and -C, and protein C (Zak et al., 1990). For example, in the case of the α -actin family the isoform present in adult heart has been found co-expressed with the skeletal isoform during the early stages of skeletal muscle development (Gunning et al., 1983; Ordahl, 1986; Ruzicka and Schwartz, 1988).

Recently we isolated and characterized the avian phospholamban gene (Toyofuku and Zak, 1991). Notably, the gene exists as a single copy and differential splicing of the gene product does not occur. Analysis of the 5'-flanking region of the gene revealed the presence of several potential muscle-specific promoter elements but the absence of several cardiac-specific sequences, which have been found in several muscle protein genes, suggesting a unique regulation program for phospholamban. Northern blot analysis of phospholamban mRNA at embryonic day 7 (stage 30–32) detected a trace amount, if any, in wing and thigh muscle (Toyofuku and Zak, 1991). At day 7 α -cardiac actin

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mRNA is still highly expressed (Hayward and Schwartz, 1986), suggesting that genetic regulation of phospholamban is not identical with regulation of α -cardiac actin in limb muscle. However, material limitations prevented analysis of earlier embryonic development by this approach.

In this work, by using *in situ* hybridization, we studied the expression of phospholamban mRNA during early avian development and compared it with that of α -cardiac and α -skeletal actin, which have been used previously in studies of avian myogenesis (Ruzicka and Schwartz, 1988). We have examined the hearts, myotomes, and limb rudiments of chick embryos in Hamburger-Hamilton stages 10 to 28+ of development. Our data demonstrate that in both cardiac and skeletal muscle phospholamban mRNA is suppressed independently of the α -actins during early myogenesis. Further, the pattern of phospholamban expression differs between myotome and muscle primordia derived from the limb bud.

RESULTS

Phospholamban mRNA During Cardiogenesis

The heart in vertebrates develops from the mesoderm which forms the ventral edges of the lateral plates (Icardo, 1984). The primitive heart tube is formed by conversion toward the embryonic midline and by subsequent fusion of paired primordia (stage 9). The heart tube becomes inflected (stage 10–11) and coiled in the shape of an S, resulting in reversal of the position of the future ventricle and future atrium (stage 12–14). The tubular heart rudiment next becomes subdivided at stage 15–24 into its four main chambers: sinus venosus, atrium, ventricle, and conus arteriosus.

Phospholamban transcripts were first detected at stage 14 of the chick embryonic heart (Figs. 2a,c). No specific signal for phospholamban above the level of background was discernable at stage 10, the next earliest stage examined (data not shown). Phospholamban mRNA signals were superimposed over the region of the epimyocardium, corresponding to the presumptive ventricle, conus arteriosus, and atrium at stage 20 (Figs. 2b,d). We found no evidence of regional differences in signal along the length of the heart. Increased phospholamban mRNA accumulation correlated with the ongoing histogenesis (stages 24 and 28+) (Fig. 3). In contrast, the endocardium showed no specific affinity for the phospholamban cDNA probes at any stage. An elevated level of silver precipitation around the aortic artery and foregut may have been due to the presence of phospholamban mRNA in smooth muscle cells (Fig. 3d). Phospholamban mRNA has been detected in smooth muscle of the pig stomach and rabbit and dog aorta, but not of the pig aorta (Raeymaekers and Jones, 1986).

Alpha-cardiac actin mRNA was first detected in stage 10 embryos in the epimyocardium of the tubular heart (data not shown). The spatial distribution of

α -cardiac actin mRNAs was largely identical to that of phospholamban mRNAs at stages 20, 24, and 28+ (Figs. 2d,f, 3). The signals of the α -cardiac actin mRNA were relatively higher than those of phospholamban mRNA at all stages analyzed. Alpha-skeletal actin mRNA was also detected in the heart; its distribution appeared to be the same as that of the other two mRNAs analyzed (data not shown).

Phospholamban mRNA During Somitogenesis and Limb Development

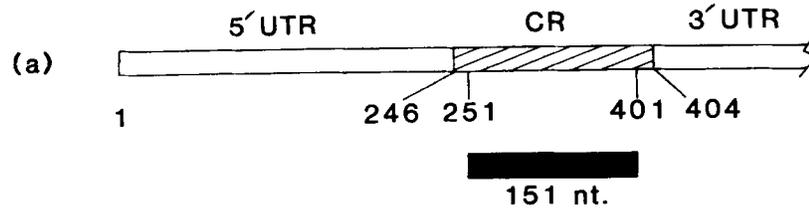
The major portion of skeletal muscle is derived from somites, which arise from segmentation of the paraxial mesoderm (Kaehn et al., 1988). The somites are first observed at stage 7, and they continue to be generated in the rostro-caudal direction. At about stage 12, the somite undergoes a compartmentalization process in which three cell layers, the dermatome, myotome, and sclerotome, are formed, and which give rise to dermis, muscle, and cartilage, respectively.

Phospholamban mRNA was first detected within the myotomal portion in the somites of stage 20 embryos (Fig. 4). Previous investigators have detected α -cardiac actin in the avian myotome as early as stage 13 (de la Brousse and Emerson, 1990). Consistent with this, we did not detect α -cardiac actin mRNA at stage 10, but did detect it in the next stage that we examined, stage 17 (Fig. 4). Thus, the expression of phospholamban appears to lag somewhat behind that of α -cardiac actin in the myotome, although the low abundance of phospholamban mRNA makes its early detection rather difficult.

To determine the localization of phospholamban and α -actin mRNAs within the myotomal portion at the time during which their large scale induction occurs, we compared sections of stage 20, 24, and 28+ embryos hybridized alternatively with phospholamban, α -cardiac actin, and α -skeletal actin probes (Figs. 4, 5). *In situ* hybridization revealed that all three mRNAs were expressed over the entire mediolateral width of the myotomal portion, and that the spatial distribution of the three mRNAs was similar.

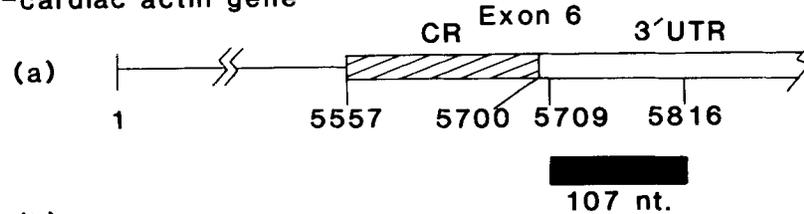
To examine the temporal and spatial expression of phospholamban and α -actin mRNAs in growing limbs, we used embryos at stages 20, 24, and 28+ (Figs. 4, 5). All three signals were very weak, if detectable at all, even up to stage 24. By stage 28+, α -cardiac and α -skeletal actin mRNA signals increased dramatically and extended in a proximal-distal gradient to the dorsal and ventral regions of the limb rudiments. These latter regions correspond to premuscle masses (Solursh and Meier, 1986). In a qualitatively similar manner, phospholamban mRNA signals also appeared in the limb rudiments and were superimposable with regions of α -actin mRNA signals. An elevated level of signal in a highly vascularized organ such as kidney (Figs. 5b,d) may have been due to the presence of smooth muscle cells.

Phospholamban cDNA



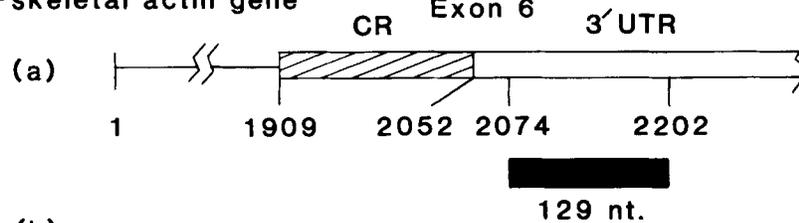
(b)

GAAGGTCCAATACATAACCCGCTCTGCTCTGAGGAGAGCCTCAACTCTTGAGGTCAACCCACAAG
CAGCCAAAGGCTCCAGGAGCTCTTTGTGAATTTCTGCCTGATCTTAATTTGCCTCTTGCTGATCT
GTATCATTGTGATGCTCCTC

 α -cardiac actin gene

(b)

CCCTACATTTACTTCCCACTCAGGATGACGACAGTATGCTTCTTGGAGTCTTCTGGCAGCCCTCC
TGAACTCCTCCGTCATTGTACAGTTTGTTTACACACTTGTGC

 α -skeletal actin gene

(b)

GCCAACCACACTCAGGATGACAATCTTGTAGGTTCCAGGCTGCTGAGGACCTGCACCAGCCATGC
AACITTTCTATTTTGTAAACAATTTCTGGTTACTGTTGCTGCAAAGCCCATGTGACACAGTGTATG

Fig. 1. Schematic representations (a) and nucleotide sequences (b) of phospholamban and of α -cardiac and α -skeletal actin probes. a: A black bar corresponds to the region of each probe. The coding region (CR) is marked as a box with oblique lines. 5' and 3' untranslated regions (UTR) are indicated by white boxes. b: 5' sense and 3' antisense primer, corresponding to the nucleotide sequences (underlined), were synthe-

sized and were used for making probes as described in Experimental Procedures. The numbering system of nucleotide sequences refers to the original papers of Toyofuku and Zak (1991) for phospholamban cDNA, Fornwald et al. (1982) for the α -cardiac actin gene, and Eldridge et al. (1985) for the α -skeletal actin gene.

Quantitation of Relative Signal Intensities

The phospholamban and both α -actin mRNA hybridization signals appeared qualitatively to follow a similar spatio-temporal pattern during development. However, when we visually compared the signal for a particular probe in the somites to the signal of that probe in the limb rudiments, it appeared that the phospholamban signal was relatively stronger in the somites than in the limbs, and that this difference was

greater than was the case for the α -actins. Inherent limitations of the method of *in situ* hybridization derive from variance in the degree of radiolabeling from probe to probe as well as variance between tissue slices, probe applications, and washing conditions. Without carefully calibrated internal control standards, it is not meaningful to quantitatively compare the intensity of the signal in one slice with that in another. However, it was possible to determine in a single slice the ratio of

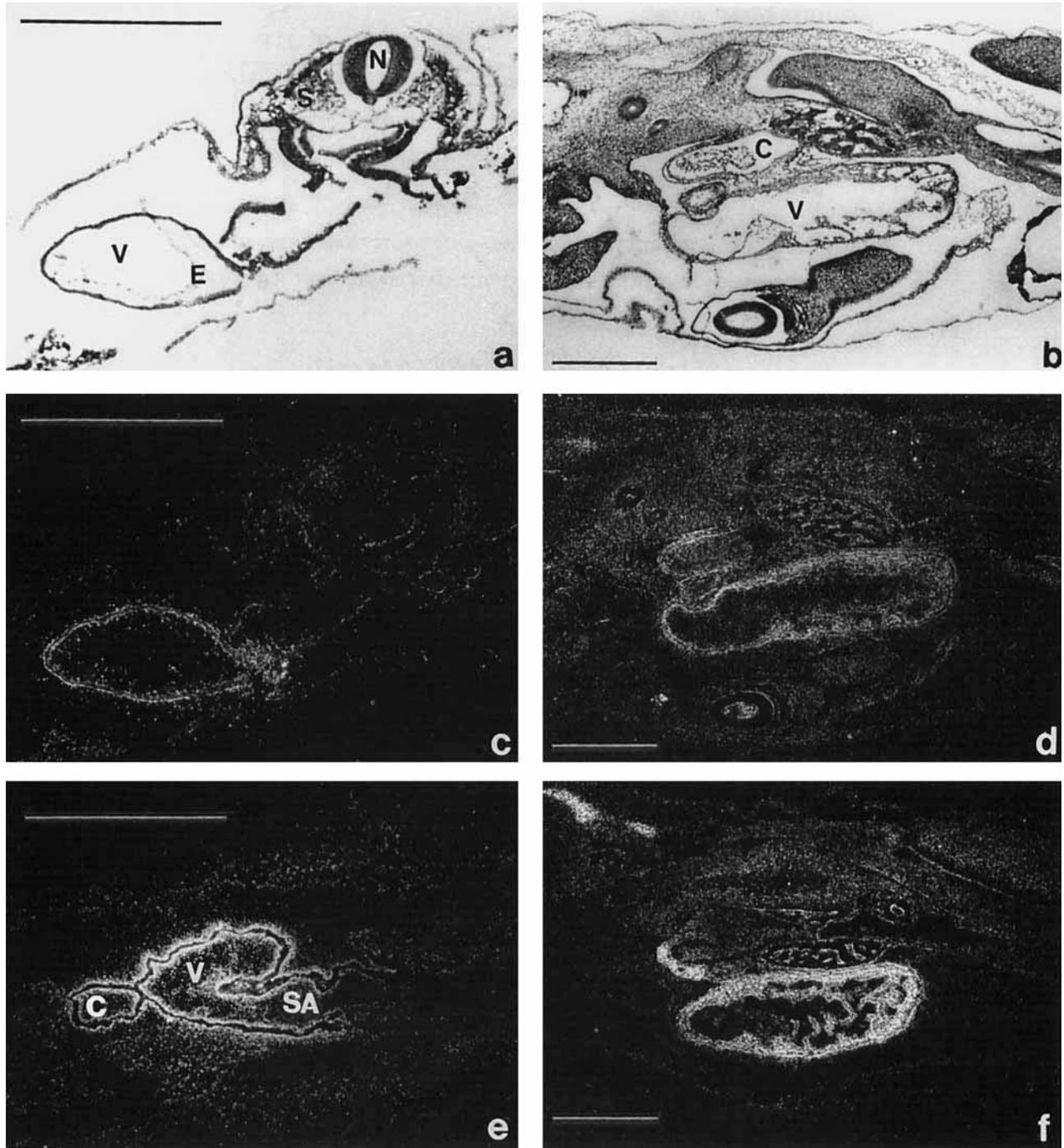


Fig. 2. Comparison of the localization of phospholamban (a–d) and α -cardiac actin (e,f) mRNAs in stage 14 (a,c,e) and stage 20 (b,d,f) chick embryos. Brightfield (a,b) and darkfield (c–f) micrographs of representative autoradiograms are shown. **a,c**: Transverse section of a stage 14 embryo through a tubular heart, hybridized with phospholamban probe. **b,d**: Fragment of a parasagittal section of a stage 20 embryo through a tubular heart, hybridized with phospholamban probe. **e**: Parasagittal section of a stage 14 embryo through the tubular heart, hybridized with

α -cardiac actin probe. **f**: Section parallel to b, hybridized with the α -cardiac actin probe. C, conus arteriosus; E, endocardium; N, neural tube; S, somite; SA, sinoatrial region; V, ventricle. In both embryonic stages, the autoradiographic signal is mostly confined to the myocardium, whereas other tissues, including somites (S) and endocardium (E), display a signal level not distinctly different from background. α -cardiac probe (e,f) resulted in stronger precipitation of silver grains than did phospholamban probe (c,d). Bar, 400 μ m.

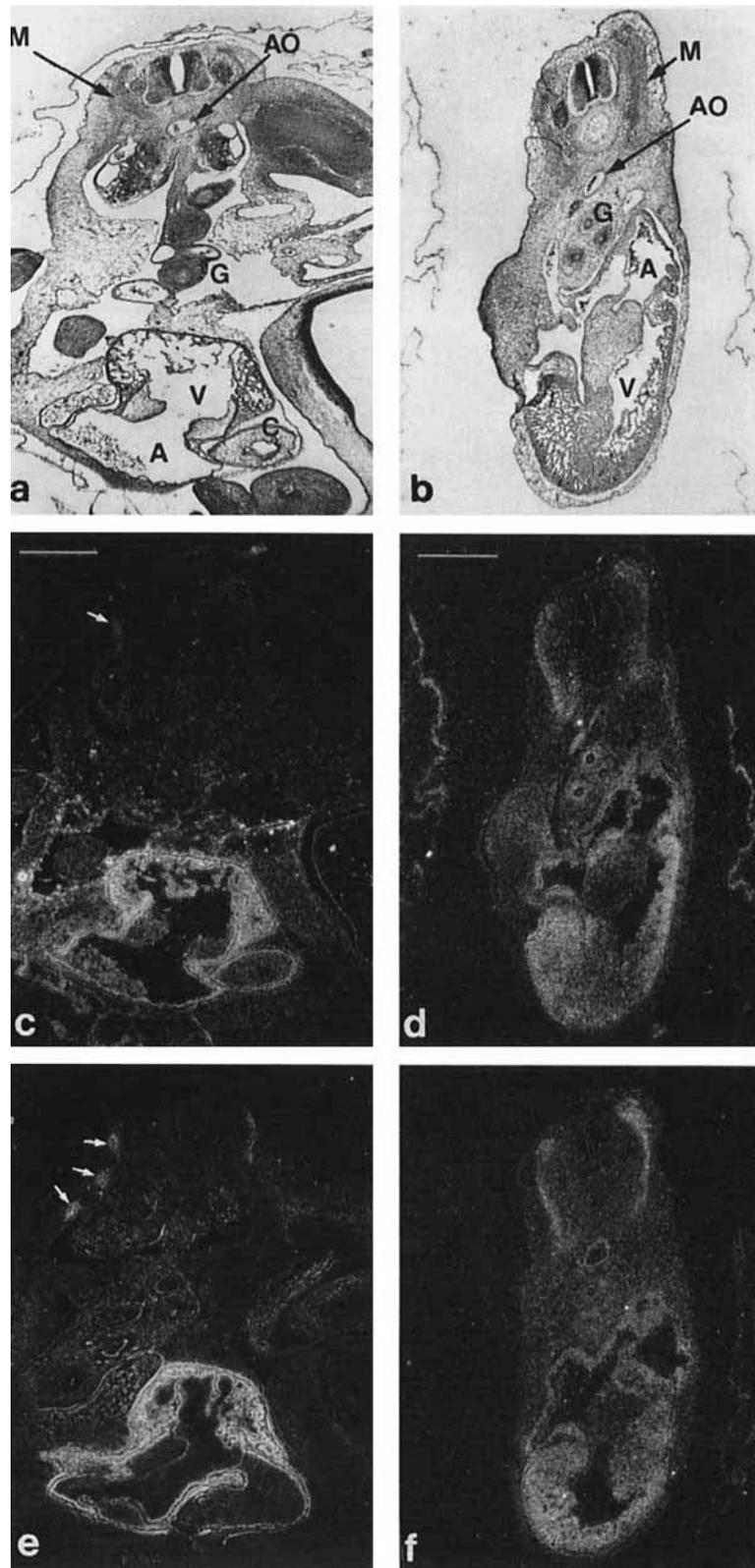


Fig. 3. Comparison of the localization of phospholamban (a-d) and α -cardiac actin (e,f) mRNAs in stage 24 (a,c,e) and stage 28+ (b,d,f) chick embryos. Brightfield (a,b) and darkfield (c-f) micrographs of the representative autoradiograms are shown. **a,c**: Fragment of a parafrontal section of a stage 24 embryo, hybridized with phospholamban probe. **b,d**: Transverse section of a stage 28+ embryo, hybridized with phospholamban probe. **e**: Section parallel to a hybridized with α -cardiac actin

probe. **f**: Section parallel to b, hybridized with α -cardiac actin probe. A, atrium; AO, aortic artery; C, conus arteriosus; G, foregut; M (in a,b; white arrows in c,e) myotome; V, ventricle. The metamerically patterned of the silver grain precipitates in the myotomal region (e) and the asymmetric pattern of the signal distribution between the right and left sides of the embryo (c-f) are the result of the angled plane of the section through the embryo. Bar, 500 μ m.

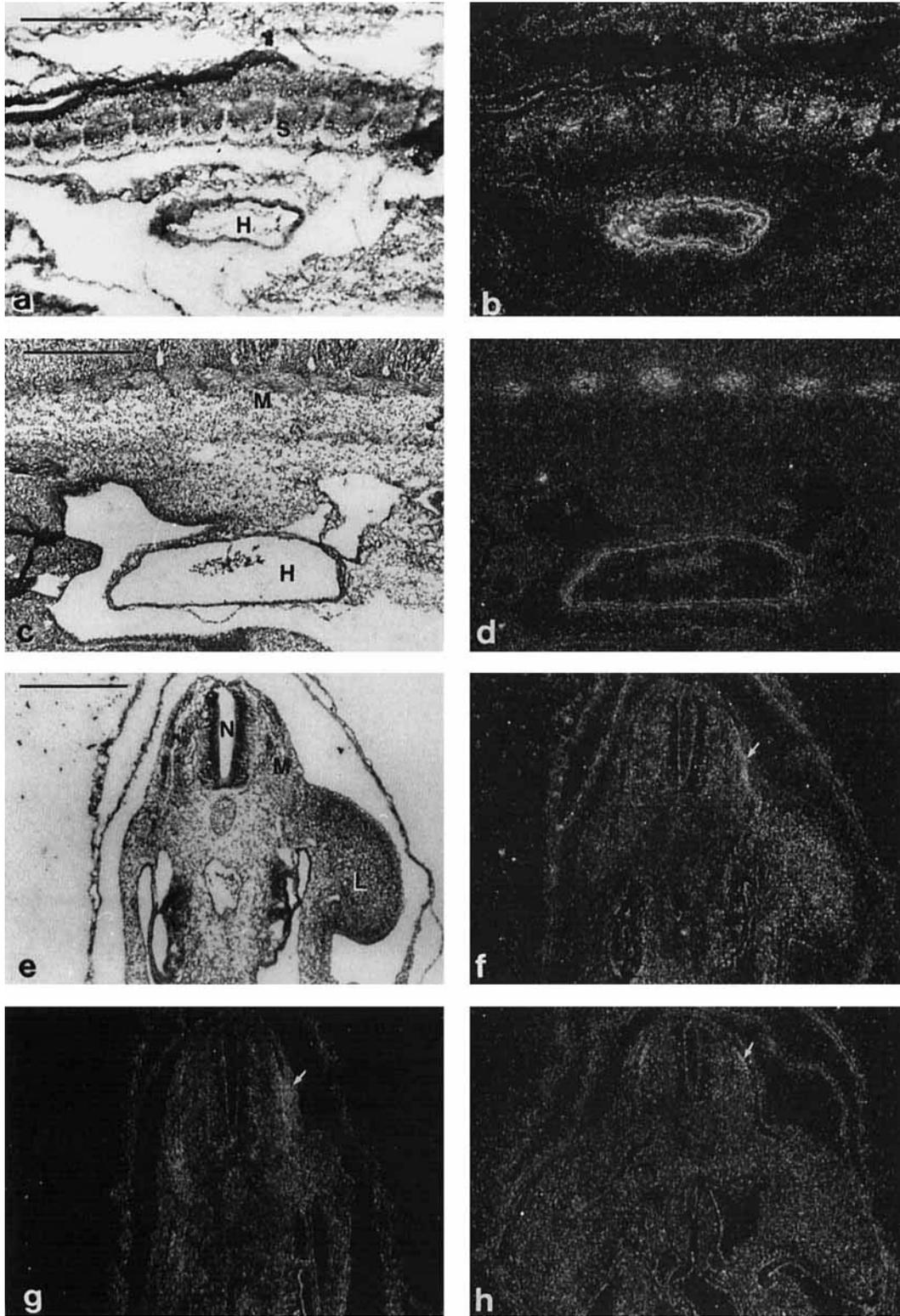


Fig. 4. Comparison of localization of phospholamban (e-f), α -cardiac actin (a-d,g), and α -skeletal actin (h) mRNAs in stage 17 (a,b) and stage 20 (c-h) chick embryo. Brightfield (a,c,e) and corresponding darkfield (b,d,f-h) micrographs of representative autoradiograms are shown. **a,b**: Fragment of a parasagittal section of a stage 17 embryo through the somites (S) and a tubular heart (H), hybridized with the α -cardiac actin probe. **c,d**: Fragment of a parasagittal section of a stage 20 embryo through the somites and a tubular heart (H), hybridized with α -cardiac actin probe. **e,f**: Brightfield and darkfield micrographs of the same fragment of a transverse section of a stage 20 embryo, hybridized with phospholamban probe. **g**: Darkfield micrograph of a corresponding fragment of a section parallel to e, hybridized with α -cardiac actin probe. **h**: Darkfield micrograph of a corresponding fragment of a section parallel to

e, hybridized with α -skeletal actin probe. H, heart; L, limb bud; M (in c,e; arrows in f-h) myotome; N, neural tube; S, somite. High levels of signal are found in the heart (a-d, H) and myotomes (a-h, M, arrows). Note that the metameric pattern of the signal in the somite region, which is very distinct in the parasagittal sections (b,d), is not seen in the transverse sections (f-h) and is marked by a single spot due to the sectioning-angle difference of 90° in relation to the sections shown in (a-d). The slight asymmetry of the pattern on the right and left sides of the embryo (f-h) is due to the angled plane of section of the embryo. The rest of the embryonic tissues, including limb rudiments (L), display low density of silver grains, close to background level. All three probes compared show a similar distribution of the signal (f-h). Bar, 300 μ m.

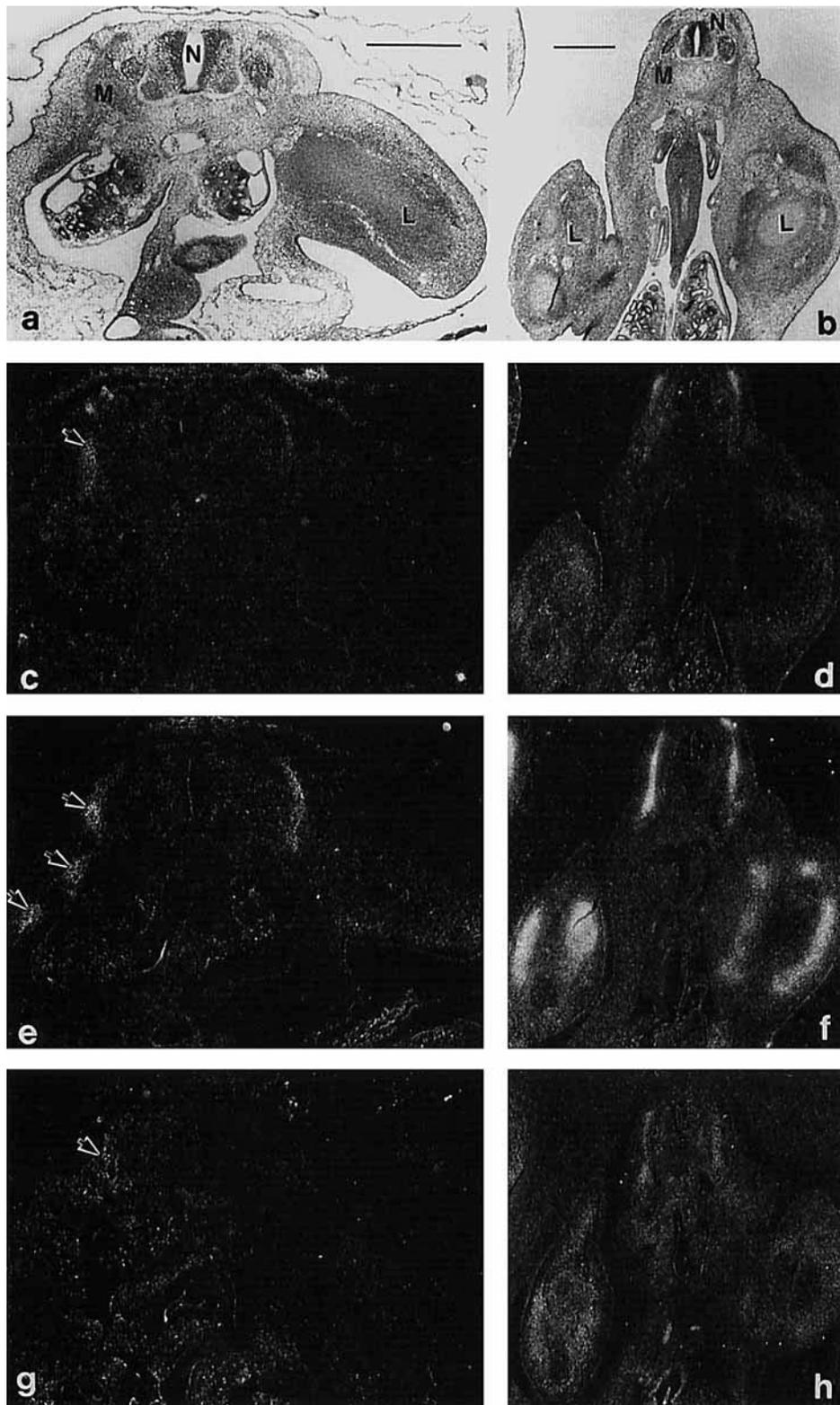


Fig. 5. Comparison of the localization of phospholamban (a-d), α -cardiac actin (e,f), and α -skeletal actin (g,h) mRNAs in stage 24 (a,c,e,g) and stage 28+ (b,d,f,h) chick embryos. Brightfield (a,b) and darkfield (c-h) micrographs of representative autoradiograms are shown. **a,c**: Fragment of a parasagittal section of a stage 24 embryo through limb rudiments, hybridized with phospholamban probe. **b,d**: Transverse section of a stage 28+ embryo through limb rudiments, hybridized with phospholamban probe. **e**: Corresponding fragment of a section parallel to a, hybridized with α -cardiac actin probe. **f**: Corresponding fragment of a section parallel to b, hybridized with α -cardiac actin probe. **g**: Corresponding fragment of a section parallel to a, hybridized with α -skeletal actin probe. **h**: Corresponding fragment of a section parallel to b, hybridized with α -skeletal actin probe. L, limb; M (in a,b; arrows in c,e,g),

myotome; N, neural tube. Note that the metameric pattern of the signal in the somite region (arrows) as well as its slight asymmetry on the right and left sides of the embryo (c-h) are due to the angled plane of section of the embryo. Although the distribution of signal is similar for all three probes, the intensity of the signal varied from probe to probe as well as between the tissues compared (see Fig. 6). The phospholamban (c,d) and α -actin (e-f) probes gave strong, though varied, signals in the myotome region of the two stages compared (24 and 28+), whereas the α -cardiac and α -skeletal actin probes showed higher levels in the muscle masses of the leg rudiments (relative to that in the myotome) than did phospholamban, especially in the stage 28+ embryo (f,h). The rest of the embryonic tissues displayed a low density of silver grains, close to background level. Bar, 400 μ m.

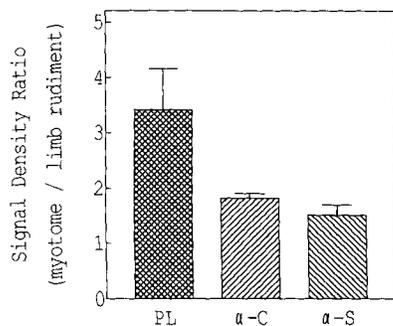


Fig. 6. Ratios of the signal density [average signal intensity per unit area ($100 \mu\text{m} \times 100 \mu\text{m}$)] in the myotome to that in the limb rudiment at stage 28+, determined for the phospholamban (PL), α -cardiac (α -C), and α -skeletal (α -S) hybridization probes. The values of the myotome and limb rudiment average signal densities (expressed as % of unit area covered by silver precipitates minus background), measured in a set of three cross-sections from the same embryo as shown in Figure 5 (d,f,h), were: PL, 3.4 and 0.55%; α -C, 4.5 and 2.3%; and α -S, 8.9 and 4.6%, respectively. For three such sets of cross-sections from the same embryo, the ratio of myotome/limb rudiment signal density [shown as averages \pm SE ($n = 3$)], were: PL, 3.41 ± 0.75 ; α -C, 1.82 ± 0.089 ; and α -S, 1.51 ± 0.19 . By ANOVA analysis, the ratio for the PL probe was significantly and marginally significantly different ($P < 0.035$, 0.051 , respectively) from the ratios for the α -S and α -C probes. The difference between the ratios obtained for the α -C and α -S probes was not significant ($P < 0.215$). The ratios for the α -C and α -S probes were significantly and marginally significantly different from 1.0 ($P < 0.006$, and 0.058), respectively.

the density of a probe signal in one tissue relative to another tissue (see Experimental Procedures).

We determined the ratio of the density of the phospholamban signal recorded from the myotome to that recorded from the limb rudiment at stage 28+, and compared it to similarly determined ratios for α -cardiac and α -skeletal actin. We found (Fig. 6) that the difference in the ratios between the two α -actins was not statistically significant. In contrast, this ratio determined for phospholamban differed from those of α -cardiac and α -skeletal actin by factors of 1.88 and 2.25, respectively. Our data also showed that the signal density for both α -actins was slightly higher in the myotome than in the limb, although to a lesser extent than determined for phospholamban.

Phospholamban and α -Actin mRNAs in Primary Cultured Cells of Pectoralis Muscle

We have investigated the expression of phospholamban and α -actin mRNAs in cultured cells from pectoralis muscle of chick fetus (embryonic day 11) and have compared it with intact heart and pectoralis muscle of the same age. The pectoralis muscle is limb bud in origin and contains mainly myoblasts committed to the fast muscle lineage (Miller and Stockdale, 1986). Northern blot analysis of total RNA extracted from native tissue showed that, whereas both α -cardiac and α -skeletal actin were detected in both heart and pectoralis muscle, phospholamban was detected in heart but not in pectoralis muscle (Fig. 7).

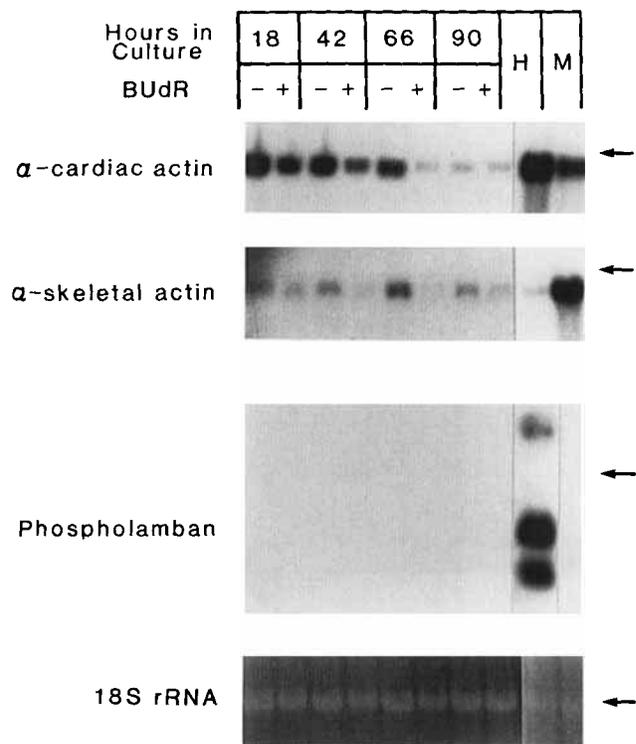


Fig. 7. Northern blot analysis of phospholamban, α -cardiac actin, α -skeletal actin mRNAs in native tissue and cultured cells. Primary cell cultures, prepared from the breast muscle of embryonic day 11 chick, were maintained without BUdR (-) or with $5 \mu\text{M}$ BUdR (+). Total RNA was isolated from cultured cells at 18, 42, 66, and 90 hr after plating, and heart (H) and breast muscle (M) of embryonic day 11 chick. Total RNA ($10 \mu\text{g}$) was size-fractionated, blotted onto nitrocellulose, and hybridized alternatively with α -cardiac actin, α -skeletal actin, and phospholamban probes. The autoradiographs were exposed for 18 hr, except for lanes H and M of α -skeletal actin and lane H of phospholamban, which were exposed for 4 hr. Ethidium bromide staining of total RNA (18S rRNA) are shown (bottom). The positions of 18S rRNA are marked with arrows.

In this study we observed the typical morphological changes in cultured myoblasts (data not shown), as demonstrated previously (Hayward and Schwartz, 1986; Lawrence et al., 1989). The primary culture 18 hr after plating consisted mainly of proliferating myogenic cells. Subsequently, myoblasts began to fuse by 38 hr, and formed multi-nucleate myotubes during 66 to 90 hr. Conversely, myoblasts grown in BUdR continued to proliferate without differentiating into myotubes.

Total RNAs were extracted from cultured cells at 18, 42, 66, 90 hr after plating. Contrary to the induction of α -actin mRNAs, phospholamban mRNAs were not detected either in proliferating myoblasts or in myotubes (Fig. 7).

DISCUSSION

We investigated the expression of phospholamban mRNA in chick embryos by using the in situ hybridization and Northern blot techniques. We detected

phospholamban mRNA in the developing heart from stage 14 and in the two major primordia of skeletal muscle—the myotomal portion of somites at stage 20 and in the limb bud at stage 24.

Initial formation of the primitive chick heart tube occurs at stage 8. At stage 9, myofibrillogenesis is detected in the caudal region and progressively more rostral region of the heart tube, coincident with muscle contraction (Hiruma and Hirakow, 1985; Hirota et al., 1985). In situ analysis using isoform-specific cDNA probes for myosin heavy chain and α -actin mRNAs in growing embryos has demonstrated their presence to be coincident with the region of myofibrillogenesis (Ruzicka and Schwartz, 1988). The constituents of the sarcoplasmic reticulum have also been found to be present in the early stage of avian heart development. By immunocytochemical method Ca^{2+} -ATPase has been shown to be present in the chick heart at stage 10 (Jorgensen and Bashir, 1984). A phosphoprotein, which was identified as phospholamban on the basis of effective phosphorylation by c-AMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent protein kinase and disaggregation into monomer of Mr 600 by heat treatment, has been shown to be present by day 4 (\pm stage 23) (Will et al., 1983). The demonstration here that phospholamban mRNA was detected in the chick ventricle at stage 14 is in agreement with the detection of phospholamban protein in the early development of the hearts of birds. However, Will et al. (1983) presented evidence that phospholamban and Ca^{2+} -ATPase in chicken are regulated independently. A discoordinate expression of phospholamban and sarcoplasmic Ca^{2+} -ATPase has also been noted by Arai et al. (1992), who detected message coding for the cardiac/slow muscle isoform of Ca^{2+} -ATPase in developing murine fast skeletal muscle while the phospholamban message was absent.

In the growing limb buds the α -actin mRNAs became strongly detectable at stage 24–28, indicating that the onset of myogenic differentiation in the limb buds occurred during this period. However, phospholamban mRNA was detected in a much lesser amount in these regions. Furthermore, phospholamban mRNA was detected in a trace amount, if any, in wing, and in thigh muscles at embryonic day 7 (stage 30–32) by Northern blot analysis (Toyofuku and Zak, 1991), whereas α -cardiac actin continues to be highly expressed in these muscles (Hayward and Schwartz, 1986). In this study we show that limb bud-derived pectoralis muscle, almost exclusively (>99%) committed to fast-type muscle fibers, at day 11 (stage 36) contained no detectable phospholamban mRNA but appreciable α -cardiac actin mRNA. In concert with the observations that in avian and mammalian models phospholamban is regulated independently of the cardiac/slow muscle Ca^{2+} -ATPase, our demonstration that phospholamban and α -cardiac actin are suppressed independently in fast muscle suggests that relative to both myofibrillar and SR constituents phospholamban is uniquely regulated.

Phospholamban mRNA was first detected in the myotomal portion of the somite, and was superimposable on the localization of α -actin mRNAs at stage 20. Its signals increased, more so relatively than in the limb buds, as the myotome developed through stage 28+. Whether the transient and low abundance of phospholamban mRNA in the limb rudiment relative to that in the myotome reflects different timing in the suppression of this cardiac/slow muscle phenotype or whether it is due to differences in the proportion or nature of lineages of myogenic cells remain to be answered. The demonstration that phospholamban expression, which in the adult is limitedly localized to slow muscle fibers, is absent in myoblasts derived from fast muscle in primary culture is consistent with the notion of different myogenic lineages. Evidence for distinct fiber-specific lineages has been obtained on the basis of the expression of fast and slow myosin isoforms (Miller and Stockdale, 1986; Crow and Stockdale, 1986). Moreover, topological heterogeneity in precursor cells within the myotome itself has also been shown to exist; muscle precursor cells in the ventral-medial quadrant of the myotome give rise in situ to the body musculature, while cells in the lateral half of the somite migrate and give rise to the muscles of the limbs (Ordahl and Le Douarin, 1992). Finally, different patterns of myosin expression have been found between primary and secondary myotubes during embryonic development of chicken leg muscle (Williams et al., 1992).

EXPERIMENTAL PROCEDURES

Animal Material

Fertilized White Leghorn chicken eggs were purchased from Sharp Sales (Wheaton, IL) and were incubated at 37°C. Developing embryos were staged on the basis of the morphologic criteria described by Hamburger and Hamilton (1951).

Preparation of Probes

mRNA isoform-specific probes were constructed from the coding region of chicken phospholamban cDNA (Toyofuku and Zak, 1991) and from 3' untranslated regions of chicken α -actin genes (Eldridge et al., 1985; Fornwald et al., 1982) (Fig. 1). The gene-specific fragments were amplified by the polymerase chain reaction with Taq DNA polymerase (Perkin Elmer Cetus). We used a digested insert from a plasmid as a template, and 5' sense and 3' antisense synthetic oligonucleotides of 20 bases as primers. The amplified double-stranded fragments were purified by agarose gel electrophoresis, and used as a template for single-stranded DNA probes by primer extension reactions with a Klenow fragment of DNA polymerase I (Pharmacia, LKB Biotechnology, Inc.), [α - ^{32}P]-dATP (6,000 Ci/mmol, Amersham), and either 5' antisense or 3' sense oligonucleotides as primers. For antisense probe, 3' antisense oligonucleotide was used as a primer; for sense probe serving as background control, 5' sense oligonu-

cleotide was used as a primer. The single-stranded ^{32}P -labeled DNA fragments were separated from unlabeled template and unincorporated free primer and nucleotides by polyacrylamide/urea gel electrophoresis. The desired fragments of approximately 100–150 bases, slightly less than the length of the unlabeled templates, were excised, eluted, and ethanol precipitated.

In Situ Hybridization Methods

Staged embryos were fixed for 30 min at room temperature in 4% paraformaldehyde in PBS (pH 7.2) containing 5 mM MgCl_2 . They were dehydrated in a series of ethanols, cleared in xylene, and embedded in Paraplast (Monoject Sci.). Tissue sections (5–10 μm thick) were cut on a conventional microtome, mounted on sterile poly-L-lysine-coated slides (ProbeOn microscopic slides, Fisher Sci.), and baked for 2 hr in a 57°C oven. Slides were stored at 4°C until in situ hybridization experiments were performed.

The in situ hybridization used in this study followed a protocol described previously (Munjaal et al., 1989). Sectioned tissues were deparaffinized in xylene and rehydrated in a series of ethanols. Samples were rinsed in PBS (pH 7.2) containing 2 mM EDTA for 2 min and digested with 1 $\mu\text{g}/\text{ml}$ proteinase K in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA for 10 min at 37°C. Samples were postfixed in 4% paraformaldehyde (pH 7.2) for 5 min, washed in 200 mM Tris-HCl, 100 mM glycine (pH 7.6) for 10 min, and treated with 0.25% (vol/vol) acetic anhydride in triethanolamine buffer (pH 8.0) for 10 min. After a rinse in $2 \times \text{SSC}$, prehybridization solution [50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, $1 \times$ Denhardt's solution, 500 $\mu\text{g}/\text{ml}$ yeast tRNA, 500 $\mu\text{g}/\text{ml}$ poly(A), 10% polyethylene glycol (MW 6000)] was applied to each sample on a slide. The slides were incubated at 37°C for 30 min and the prehybridization solution was drained. Hybridization solutions [50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, $1 \times$ Denhardt's solution, 500 $\mu\text{g}/\text{ml}$ yeast tRNA, 500 $\mu\text{g}/\text{ml}$ poly(A), 10% polyethylene glycol (MW 6000), 20% dextran sulfate] with 5×10^4 cpm/ μl ^{32}P -labeled single-stranded DNA probes were applied to the slides. Sections were sandwiched with sterile slides (ProbeOn microscopic slides, Fisher Sci.) and covered with Parafilm. The slides were transferred to a 37°C incubator and incubated for 6 hr. After hybridization, slides were detached and washed at room temperature in a large volume of $2 \times \text{SSC}$ for 1 hr followed by 50% formamide, $2 \times \text{SSC}$ for 40 min at 37°C, and 50% formamide, $1 \times \text{SSC}$ for 20 min at 37°C. A final wash in $1 \times \text{SSC}$ was performed at room temperature. The slides were dehydrated through a series of ethanols (70–95%) containing 50 mM ammonium acetate and then air dried. Hybridization signals were localized by autoradiography. Slides were dipped in Kodak NTB-2 nuclear track emulsion with a Pelco emulsion coater (Ted Pella, Inc.). After 10 days of exposure, sections

were developed and stained with hematoxylin and eosin. Coverslips were mounted in Permount.

Quantitation of Relative In Situ Signal Intensities

Measurements were made with a Cue-2 Image Analyzer, Version 3.0 (Olympus), interfaced with an Olympus BH-2 microscope via closed-circuit television (Sony Galai CCD Video Camera, Sony Trinitron Color Video Monitor). The Cue-2 system consisted of a video chain, a high speed digital image processor, and a general purpose computer (Zeos 386, Zeos International, Ltd.) with associated peripherals. The video signal from the television camera was digitized and transferred simultaneously to the digital image processor and to a television monitor. The processed image was displayed on the Sony monitor and transferred to the computer for quantitative analysis. Brightfield optics were used for locating and orienting the specimen, whereas a dark-field condenser with a $40\times$ Olympus SPlan objective lens was used for the actual measurements. Thresholds of the image analyzer were established over a randomly selected tissue section so that the accuracy of grain distinction from the underlying tissue and the grain resolution were maximized. Within a pre-set square counting region ($100 \times 100 \mu\text{m}$) defined and calibrated by the operator, the surface area occupied by the silver precipitates was automatically determined. Measurement of the surface area of the precipitates, rather than grain density, was used for quantitative evaluation of the signal because, in areas heavily loaded with silver, individual grains could not be distinguished due to their overlap.

For a given probe, we measured the total signal in the myotome and in the limb rudiment in a single cross-section of an HH stage 28+ embryo and calculated the signal density [i.e., the average signal intensity per unit area ($100 \mu\text{m} \times 100 \mu\text{m}$)] for each tissue. Three sets of three parallel transverse sections of the same embryo were selected, and each set was exposed to a probe specific either for phospholamban, or for α -cardiac or α -skeletal actin. In each section, 5–7 unit areas covered the entire area of one myotome cross-section, and about 50 unit areas entirely covered the premuscle mass of the leg cross-section. Separate measurements were made on the cross-section through the notochord (next to the myotome) and on the cross-section through the bone rudiment (within the leg). We subtracted the average values measured in notochord and bone to obtain the specific signal density in the myotome and the leg, respectively, in order to include possible non-specific tissue-related signal in measurements of the background values. For example, for the case of the least signal density measured, that of the phospholamban probe in the stage 28+ limb bud, as in Figure 5d, the average value of a unit area covered by silver precipitates for the region of bone rudiment in three specimens was $.296 \pm .014\%$. The average value for the region of premuscle mass was $.752 \pm .049\%$.

Cell Cultures

Primary cultures were prepared from the breast muscles of chick (embryonic day 11) essentially as described previously (Clark and Fischman, 1983). Briefly, the muscles were dissected and minced, and the cells were dissociated with 0.125% trypsin and 0.0125% collagenase. To reduce the number of fibroblasts, the myoblasts were preplated twice on nongelatinized culture plates. The cells were filtered through sterile lens paper for removal of aggregates and plated on gelatin-coated Petri dishes (Falcon brand, Becton Dickinson Labware) at a density of 8×10^5 cells/60 mm culture dish and grown at 37°C in basal medium Eagle (GIBCO-BRL) containing 10% horse serum and 2% chicken embryo extract, with 10 U/ml penicillin and 10 µg/ml streptomycin. For the experiments involving BUdR, the cultures were maintained continuously in medium containing 10 µM BUdR.

RNA Isolation and Northern Blots

Total RNA was isolated by guanidine thiocyanate extraction (Chirgwin et al., 1979). RNAs were electrophoresed on 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose (Schleicher & Schuell, Inc). The blot was prehybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.05% sodium pyrophosphate, and 500 µg/ml salmon sperm DNA at 42°C for 4 hr, and hybridized in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 0.05% sodium pyrophosphate, and 50 µg/ml salmon sperm DNA with ³²P-labeled single-stranded DNA probes at 42°C for 12 hr. The blots were washed twice in 2 × SSC, 0.1% SDS at room temperature and then one time in 1 × SSC, 0.1% SDS at 55°C for 15 min. The blots were exposed to Kodak XAR film for appropriate periods.

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